Universidad de Navarra

ESTUDIO DE LA TOXICIDAD COMBINADA DE AFLATOXINA B1 Y OCRATOXINA A EN MODELOS *IN VITRO* E *IN VIVO*

COMBINED TOXICITY OF AFLATOXIN B1 AND OCHRATOXIN A IN *IN VITRO* AND *IN VIVO* MODELS

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que presenta Dña. Laura Ana Corcuera Martínez para aspirar al grado de Doctor por la Universidad de Navarra, ha sido realizado en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología bajo la dirección de la Dra. Adela López de Cerain Salsamendi y la co-dirección de la Dra. Elena González-Peñas

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"Lo consiguieron porque no sabían que era imposible"

Jean Cocteau

ABREVIATURAS /ABBREVIATIONS

3-ADON: 3-acetildeoxinivalenol / 3-acetyldeoxynivalenol

8-oxo-dG: 8-oxoguanina / 8-oxoguanine

15-ADON: 15-acetildeoxinivalenol / 15-acetyldeoxynivalenol

AFB1: Aflatoxina B1 / Aflatoxin B1

AFB1-FAPY: AFB1-formamidopirimidina / AFB1-formamidopyrimidin

AFB2: Aflatoxina B2 / Aflatoxin B2

AFBO: AFB1-exo-8,9-epóxido / AFB1-exo-8,9-epoxide

AFG1: Aflatoxina G1 / Aflatoxin G1

AFG2: Aflatoxina G2 / Aflatoxin G2

AEFI: Asociación Española de Farmacéuticos de la Industria

AOAC: Asociación Internacional de Químicos Analistas / Association of Official Analytical Chemists

AP site: Apirimidinic or apurinic site

AU: Arbitrary units

bw: Body weight

CIT: Citrinina /Citrinin

CV: Coeficiente de variación

DCFH-DA: Diacetato de dihidrodiclorofluoresceína / Dihydrodichlorofluorescein diacetate

DL_{50}: Dosis letal 50

DON: Deoxinivalenol / Deoxynivalenol

DTI: Daily tolerable intake

EC: Comisión Europea / European Commision

EFSA: Autoridad Europea de Seguridad Alimentaria / European Food Safety Authority

Endo III: Endonucleasa III / Endonuclease III

FPG: Formamidopirimidina ADN glicosilasa / Formamidopyrimidine DNA glycosylase

FAO/OIEA (IAEA): Organización de las Naciones Unidas para la Agricultura y la Alimentación / Organismo Internacional de Energía Atómica / Food and Agriculture Organization of the United Nations / International Atomic Energy Agency

FAO/OMS: Organización de las Naciones Unidas para la Agricultura y la Alimentación Organización Mundial de la Salud / Food and Agriculture Organization of the United Nations/World Health Organization

FDA: Agencia de medicamentos y alimentos / Food and Drug Administration

FLD: Detector de fluorescencia / Fluorescence detector

FB1: Fumonisina B1 / Fumonisin B1

GST: Complejo glutatión–S-transferasa / Glutation-S-transferase complex

HPLC: Cromatografía líquida de alta resolución / High Performance Liquid Chromatography

HT-2: Toxina HT-2 /HT-2 toxin

IARC: Agencia Internacional para la Investigación sobre el Cáncer / International Agency for the Research on Cancer

ICH: Conferencia Internacional de Armonización / International Conference on Harmonisation

IDT: Ingesta diaria tolerable

JEFCA: Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios / Joint FAO/WHO Expert Committee on Food Additives

LD_{50}: Lethal Dose 50

LD/LOD: Límite de detección / Limit of detection

LC/LOQ: Límite de cuantificación / Limit of quantification

NIV: Nivalenol /Nivalenol

NTP: Programa Nacional de Toxicología / National Toxicology Program

OECD: Organización para la Cooperación y el Desarrollo Económico / Organisation for the Economics Co-operation and Development

OTA: Ocratoxina A / Ochratoxin A

PBS: Tampón fosfato / Phosphate buffered saline

pc: Peso corporal

PECE: Extracto de cacao enriquecido en polifenoles / polyphenol-enriched cocoa extract

pK_a: Constante de disociación ácida / Acid dissociation constant

RE: Relative error

SCF: Comité Científico de la Alimentación de la Comisión Europea / Scientific Committee of Food

SCOOP: Cooperación científica sobre las cuestiones relativas a la alimentación / Scientific Co-operation on Questions relating to Food)

RF: Relative fluorescence

ROS: Especies reactivas de oxígeno / Reactive oxygen species

RSD: Desviación estándar / Relative standard deviation

SE: Standard error

$t_{1/2}$: Semivida de eliminación /Elimination half life

T-2: Toxina T-2/ T-2 toxin

TWI: Tolerable weekly intake

UA: Unidades arbitrarias

UHPLC-LD: Cromatografía líquida de ultra alta resolución con detector ultravioleta / Ultra high performance liquid chromatography-fluorescence detector

UV: Ultraviolet detector

WHO: Organización Mundial de la Salud / World Health Organisation

ZEA: Zearalenona / Zearalenone

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Capítulo 1 / Chapter 1

Introducción general / General introduction

MICOTOXINAS

Desde hace siglos el hombre ha empleado los hongos que se desarrollan en los alimentos para obtener otros alimentos de características organolépticas distintas al original. Así los hongos se empleaban para la elaboración de vinos, quesos, cervezas, embutidos y también en la producción de fármacos. Sin embargo, algunos de los hongos que crecen sobre los materiales vegetales producen toxinas con efectos indeseables para plantas y animales.

Micotoxina es un término que deriva del griego antiguo *mykes, mukos* (μύκης), que significa hongo, y del latín *toxicum*, que significa veneno. Las micotoxinas son metabolitos secundarios con distintas propiedades químicas, biológicas y toxicológicas, producidas por hongos que se desarrollan en productos vegetales.

Se denomina micotoxicosis a la intoxicación provocada por una micotoxina y pueden darse intoxicaciones agudas o crónicas. Generalmente, el riesgo de intoxicación aguda por micotoxinas es bajo o moderado en comparación con intoxicaciones de origen microbiológico o por contaminantes químicos. Es la exposición crónica la de mayor relevancia en el hombre; ya que son ingeridas por el ser humano asociadas a los alimentos, en pequeñas dosis y durante periodos de tiempo prolongados (Soriano del Castillo, 2007). En particular, es especialmente relevante la ingesta crónica de aquellas micotoxinas que presentan efectos cancerígenos y/o inmunosupresores.

Los primeros casos de micotoxicosis comenzaron a documentarse en la Edad Media por la contaminación del centeno con el hongo *Claviceps purpurea*, vulgarmente llamado Cornezuelo del centeno. Los alcaloides generados sobre el cereal provocan la enfermedad llamada ergotismo o fuego de San Antonio. Esta micotoxicosis ya era conocida en la antigüedad por los romanos, que la denominaban *ignis sacer* o *ignis martialis*, fuego sagrado o fuego sacro, por sus síntomas de ataques, convulsiones, alucinaciones, inflamaciones y gangrenas (FAO/OIEA, 2003). Las micotoxicosis adquirieron mayor interés en 1960, cuando murieron en Inglaterra pavos, patos y otros animales de granja a causa de una enfermedad denominada "Enfermedad X de los pavos". Fue provocada por contaminación con el hongo *Aspergillus flavus* en harinas de cacahuete para piensos importadas de Brasil (Soriano del Castillo, 2007; Austwick, 1978), y supuso importantes pérdidas en el sector agroalimentario.

Muchas especies de hongos infectan los cultivos y producen las micotoxinas en los productos vegetales antes de su recolección, denominándose hongos de campo. Otras especies, denominadas hongos de almacenamiento, se desarrollan, generan micotoxinas o incrementan su síntesis cuando el producto se ha recolectado y las condiciones de almacenamiento lo permiten; y otras son capaces de producirlas en ambas condiciones. Fundamentalmente, las micotoxinas son producidas por tres géneros de hongos: *Aspergillus, Fusarium* y *Penicillium*. *Fusarium* es mayoritariamente hongo de campo, *Penicillium* de almacenamiento y *Aspergillus* es micotoxigénico en ambos medios (Soriano del Castillo, 2007). En un alimento, la ausencia de especies potencialmente toxigénicas no indica que no pueda contener micotoxinas. Los hongos presentes pueden haber dejado de ser viables después de un tratamiento tecnológico aplicado para su elaboración (calor, por ejemplo), pero las micotoxinas, debido a su termoresistencia, pueden encontrarse aún presentes en el mismo (Maganen y Olsen, 2004).

Según la FAO, las micotoxinas son objeto de interés mundial debido a las importantes pérdidas económicas que acarrean sus efectos sobre la salud de las personas, la productividad de los animales y el comercio nacional e internacional. Una micotoxina se considera "importante" si se ha demostrado su capacidad de provocar efectos adversos considerables en varios países (FAO/OIEA, 2003). Las micotoxinas que tienen mayor importancia desde el punto de vista agroalimentario son las aflatoxinas (B, G y M), la citrinina, las fumonisinas (especialmente FB1), la ocratoxina A, la patulina, los tricotecenos (DAS, T-2, HT-2, NEO, DON, NIV y fusarenona X) y la zearalenona. Éstas son capaces de producir su efecto tóxico a concentraciones extremadamente bajas (μg/L o μg/kg) (Pascual Anderson, 2005). De entre sus posibles efectos tóxicos, es muy importante su efecto inmunosupresor, ya que pueden disminuir las defensas, tanto de animales como de humanos, aumentando la susceptibilidad a infecciones (Sharma, 1993). La Agencia Internacional de Investigación sobre el Cáncer (IARC) ha clasificado varias micotoxinas como carcinógenas o potencialmente carcinógenas para el hombre en función de evidencias experimentales y epidemiológicas. En la tabla 1 se presenta un breve resumen de los principales efectos tóxicos de las micotoxinas más relevantes.

Tabla 1: Principales efectos tóxicos de las micotoxinas con mayor prevalencia agroalimentaria.

Micotoxina	Efectos Fisiopatológicos	Clasificación IARC	Referencias
Aflatoxinas	Daño hepático agudo, cirrosis, inducción de tumores, inmunosupresivas, teratogénicas, carcinogénica	B y G: 1 M1: 2B	IARC, 1987 IARC, 1993
Citrinina	Toxicidad renal, temblores corporales, inmunosupresiva	3	IARC, 1987 Soriano del Castillo, 2007
Fumonisinas	Leucoencefalomalacia, edema pulmonar, cáncer de esófago, hepatotóxica, embriotóxica, teratogénica	B1: 2B	IARC, 1993 WHO/IPCS, 2000
Ocratoxinas	Nefropatía endémica de los Balcanes, acumulación en riñon, tubulonefritis, vómitos, teratogénica, mutagénica, embriotóxica	A: 2B	IARC, 2002
Patulina	Trastornos gastrointestinales y neurológicos, temblores corporales, mutágena e inductora de tumores	3	IARC, 1987 Soriano del Castillo, 2007
Tricotecenos	Anorexia, emesis, dolor abdominal, diarrea, inmunosupresión	3	IARC, 1993 FAO/OIEA, 2003
Zearalenona	Efectos estrogénicos (alteraciones en niveles de progesterona y estradiol), descenso fertilidad, alteraciones en glándulas suprarrenales, tiroides y pituitaria	3	IARC, 1993 FAO/OIEA, 2003

Clasificación IARC: grupo 1: carcinógeno para humanos, grupo 2A: probable carcinógeno para humanos, grupo 2B: posible carcinógeno para humanos, grupo 3: no clasificable como carcinógeno para humanos, grupo 4: probablemente no es carcinógeno para humanos.

AFLATOXINA B1

Propiedades químicas

La AFB1 pertenece a la familia de las difurano cumarinas. Su nomenclatura sistemática es 2,3,6aα,9aα-tetrahidro-4-metoxiciclopenta[c]-b-furo[2',3':4,5]furo[2,3-h]cromeno-1,11-diona, $C_{17}H_{12}O_6$ es su fórmula molecular es y su peso es 312,27 g/mol. Ligeras variaciones de su estructura química dan lugar al conjunto de las aflatoxinas que pueden encontrarse en la naturaleza (AFB1, AFB2, AFG1 y AFG2). La AFB1 es la que se encuentra en mayor concentración, seguida de AFG1, AFB2 y AFG2. El orden que siguen estas sustancias con respecto a su toxicidad aguda y crónica es AFB1>AFG1>AFB2>AFG2 y está directamente relacionado con la capacidad de formar un epóxido en el doble enlace 8-9 (marcado con una flecha roja en la figura) y la potencia asociada a los anillos de ciclopentenona (flecha azul) (McLean y Dutton, 1995). Las aflatoxinas M1 y M2 son los productos de hidroxilación del metabolismo oxidativo de las aflatoxinas B1 y B2, respectivamente.

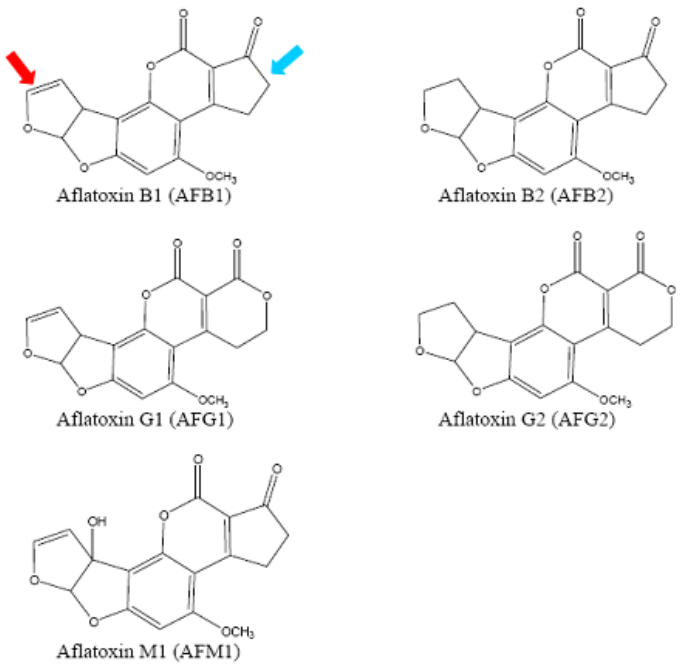


Figura 1: Aflatoxinas presentes en la naturaleza (EFSA, 2007)

Son inodoras, insípidas, incoloras e inestables en estado puro en contacto con la luz y el aire. Sufren hidrólisis alcalina (pH> 10,5 de amoniaco o lejía) y se degradan en solución ácida pH<3. La AFB1 es poco soluble en agua (10-30 μg/mL), pero se disuelve bien en soluciones acuosas de metanol, dimetilsulfóxido, cloroformo, acetona y acetonitrilo. Estas características lipófilas facilitan su paso a través de las membranas celulares y su bioacumulación (Palanee *et al.*, 2001).

Micología

Las aflatoxinas son producidas por tres clases de hongos del género *Aspergillus,* fundamentalmente: *A. flavus*, *A. parasiticus* y *A. nomius*. El porcentaje de cepas capaces de producir aflatoxinas depende, no sólo del genotipo, sino también de los factores ambientales que influirán en el crecimiento y metabolismo del hongo. Aproximadamente un 40% de las cepas de *A. flavus* son aflatoxigénicas y dependientes de la estación y lugar geográfico. Sin embargo, aproximadamente el 100% de las cepas de *A. parasiticus* y *A. nomius* son aflatoxigénicas (Soriano del Castillo, 2007). La presencia y contaminación de aflatoxinas no sólo depende de factores geográficos y estacionales, sino también de las condiciones de cultivo, recolección y almacenamiento. Los hongos aflatoxigénicos pueden infectar los cultivos y producir la toxina en cualquiera de los tres pasos anteriores si se dan las condiciones adecuadas para el hongo.

Prevalencia en alimentos, legislación y exposición

Las aflatoxinas se han detectado como contaminantes naturales en productos agrícolas, habiéndose confirmado su presencia en prácticamente todas las zonas del mundo y, en mayor o menor medida, en todos los alimentos de primera necesidad (Soriano del Castillo, 2007). Los alimentos típicamente contaminados con AFB1 son maíz, cacahuetes, pistachos, nueces de Brasil y semillas de algodón. También puede aparecer en semillas oleaginosas como el girasol, la soja, aceites vegetales sin refinar, y otros frutos secos como almendras, avellanas o nueces. También en especias como pimentón, chili, pimienta, etc. Puede encontrarse en frutas desecadas, como higos y pasas, en cacao, café y en cereales y productos derivados. También es relevante su presencia en piensos de alimentación animal, y pueden encontrarse en huevos, sangre, vísceras, etc. Aunque la tasa de transferencia entre el pienso y los productos comestibles de origen animal suele ser baja. En los alimentos, las aflatoxinas son químicamente estables y resistentes a la degradación bajo procedimientos de cocción normales (termoestables); por lo tanto, una vez que se producen son difíciles de eliminar de los alimentos (Soriano del Castillo, 2007).

Desde 1999 existe legislación en la Unión Europea que regula el contenido máximo de aflatoxinas en productos vegetales. Cacahuetes, nueces, frutos secos, cereales y productos procesados para el consumo no deben estar contaminados con más de 4 μg/kg de aflatoxinas (AFB1+B2+G1+G2), y no más de 2 μg/kg de AFB1. Para especias, los límites son algo superiores, con un máximo de 10 μg/kg de aflatoxinas y 5 μg/kg de AFB1. La AFB1 se metaboliza a AFM1 en rumiantes alimentados con piensos contaminados, por tanto en leche y derivados lácteos se ha establecido un límite máximo de 0,05 μg/kg de AFM1. Para alimentos destinados al consumo infantil no se permiten niveles superiores a 0,10 μg/kg de AFB1 y 0,025 μg/kg de AFM1 (EC, 2006).

La Unión Europea ha establecido grupos de cooperación científica (SCOOP) para recoger datos sobre la presencia de micotoxinas en los alimentos y valorar la ingesta diaria de dichas toxinas por la población de los Estados miembros. Para la AFB1 se ha estimado un consumo medio diario de 0,03 a 1,3 ng/kg de pc (EC, 1997a). Sin embargo, grandes consumidores de alimentos que suelen aparecer contaminados con aflatoxinas, como los frutos secos, pueden ver aumentada su exposición diaria a AFB1 hasta 2,3 ng/kg de pc (EFSA, 2007). Debido a la alta toxicidad de la AFB1 no se han podido establecer límites de exposición segura, por ello debe aplicarse el principio ALARA (as low as reasonably achievable), que intenta mantener los límites de exposición tan bajos como sea posible.

Metabolismo

La exposición humana a aflatoxinas se produce principalmente por ingestión de alimentos contaminados, aunque puede inhalarse ocasionalmente debido a la exposición laboral. Una vez en el organismo se absorben por el tracto gastrointestinal gracias a su alta liposolubilidad. Llegan al hígado, donde comienza su metabolismo, por circulación portal. En humanos, la AFB1 sigue un modelo cinético bicompartimental con una fase de absorción y distribución rápida y una fase de eliminación más lenta (EFSA, 2007; Jubert *et al.*, 2009). La vida media plasmática varía entre especies (Wong y Hsieh, 1980), y aproximadamente, el 95% de la dosis administrada se elimina en 24 h (Jubert *et al.*, 2009).

La AFB1 es biotransformada en el hígado por monooxigenasas microsomales de función mixta de la superfamilia del citocromo P450 (CYP1A2, 3A4, 3A5 y 3A7) (figura 2) (McLean y Dutton, 1995). El CYP3A4 interviene en la formación de la AFQ1 y de la forma exo-epóxido de la AFB1, que es capaz de unirse al ADN. El CYP1A2 forma la AFM1 y la forma endo-epóxido (que no se une al ADN) (EFSA, 2007; Urrego Novoa y Díaz, 2006). Es importante destacar que existen

grandes diferencias interespecíficas en el metabolismo de la AFB1. Estas diferencias se deben a la afinidad y actividad catalítica de los CYPs y de la detoxificación del complejo glutatión –S-transferasa (GST) entre las distintas especies, así como de la capacidad de algunos CYPs de ser inducidos. En contraste con experimentos en roedores, se ha evidenciado que en humanos el CYP3A4 (el predominante) tiene una menor afinidad por la AFB1, y en cambio CYP1A2 posee alta afinidad. Previsiblemente, a concentraciones de AFB1 bajas en alimentos, ésta será biotransformada preferentemente por el CYP1A2; mientras que a concentraciones altas, la AFB1 será bioactivada por el CYP3A4 (EFSA, 2007).

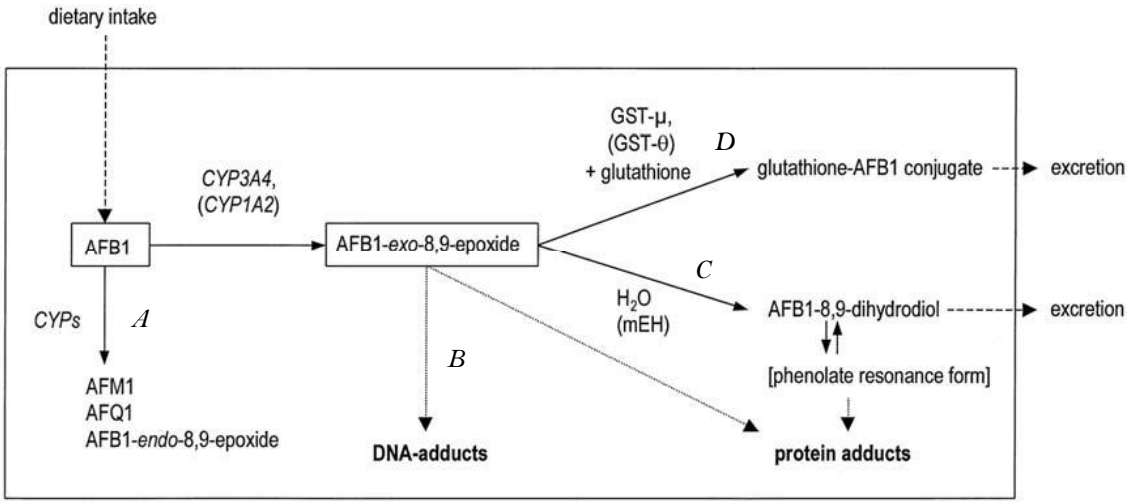


Figura 2: Metabolitos de la AFB1 (EFSA, 2007)

La AFB1 puede convertirse, de forma reversible, por la NADPH-reductasa en aflatoxicol, que actúa como reservorio de AFB1. La AFM1, AFQ1 y el endo epóxido de la AFB1 (figura 2, A) pueden ser eliminadas por los hepatocitos, mientras que el epóxido se une a ácidos nucleicos y proteínas (figura 2, B). Dicho epóxido puede hidratarse a su dihidrodiol (8,9-dihidro-8,9-dihidroxi AFB1) (figura 2, C), reorganizándose después en el correspondiente dialdehído y condensar con aminoácidos o proteínas (como la albúmina) formando bases de Shiff (iminas C=N). El producto de hidrólisis de la AFB1 es la AFB2a, que también es capaz de unirse a proteínas y formar bases de Shiff (McLean y Dutton, 1995). La vía de detoxificación principal de la AFB1 es la conjugación del epóxido con glutatión mediado por la glutation S-transferasa (GST) (figura 2, D), aunque también es posible la actuación de la UDP-glucoronil-transferasa, sulfotransferasas (McLean y Dutton, 1995). La mayoría de los metabolitos de la AFB1 (AFM1, AFQ1 y AFP1) son excretados por la orina en su

forma conjugada con glucorónidos o sulfatos. También puede darse excreción biliar, donde se rompe el conjugado de AFB1 y se reabsorbe apareciendo recirculación entero-hepática de la micotoxina. Una vía muy importante de excreción es la del metabolito genotóxico AFM1 por la leche, consistiendo una vía de alto riesgo para los lactantes (EFSA, 2007). La AFM1 se excreta en 48 h y representa entre el 1-4% de la AFB1 ingerida (Urrego Novoa y Díaz, 2006).

Toxicidad general

La AFB1 causa hepatotoxicidad aguda en humanos y animales de experimentación. Los síntomas son necrosis hemorrágica en el hígado, proliferación de los conductos biliares, edema y letargo, y, en intoxicaciones muy severas, la muerte. Los estudios en animales han encontrado dos órdenes de magnitud en la LD_{50} media para la AFB1: conejos y patos son muy sensibles con LD_{50} media de 0,3 mg/kg pc, mientras que pollos, ratas, ratones y monos tienen una LD_{50} media de 18 mg/kg pc. Wild y Gong (2010) estimaron una DL_{50} en humanos de entre 0,6 y 1,6 mg kg pc a partir de datos de víctimas en Kenia e India, encontrándonos, por lo tanto, en resistencia comparable a la de los conejos o perros, pero menor que la de los roedores. Los seres humanos adultos tienen mayor tolerancia a las intoxicaciones agudas de aflatoxina, y, por lo general, las víctimas mortales, si se dan, suelen ser niños (EFSA, 2007; Williams *et al.*, 2004). Las aflatoxicosis agudas se han relacionado con el consumo de maíz y no con el de frutos secos. Ésto puede ser el reflejo de la susceptibilidad del maíz a la contaminación con aflatoxinas y la alta ingesta diaria de este cereal en los países en vías de desarrollo (Wild y Gong, 2010).

La AFB1 también es capaz de afectar al sistema inmune, produciendo en animales de experimentación aplasias en el timo, reducción de la función y número de linfocitos T y reducción de la actividad fagocítica y del complemento (Williams *et al.*, 2004). Estudios en niños de Gambia y Ghana indicaron que la exposición alimentaria a AFB1 reducía la actividad inmunológica, afectando la capacidad de los individuos a resistir infecciones; y estudios recientes en niños menores de 5 años de Benin y Togo han relacionado la exposición a AFB1 con retrasos en el crecimiento y falta de peso (EFSA, 2007).

Genotoxicidad: mecanismos moleculares

La Agencia Internacional para la Investigación del Cáncer (IARC) ha concluido que existe suficiente evidencia de que estas micotoxinas son carcinógenas para el ser humano (grupo 1), estando

involucradas en el cáncer de hígado, sobre todo en pacientes portadores de antígenos del virus de la Hepatitis B (IARC, 1987; IARC, 1993). En animales de experimentación hay suficiente evidencia de carcinogenicidad de las mezclas naturales de aflatoxinas, así como de las aflatoxinas B1, G1 y M1, limitada evidencia para la aflatoxina B2 y evidencia inadecuada para la aflatoxina G2. Las aflatoxinas son capaces de formar tumores en el hígado y en otros órganos, principalmente riñón y colon. La AFB1 es genotóxica *in vitro* e *in vivo* (EFSA, 2007). El comité conjunto de expertos FAO/WHO en aditivos alimentarios (JECFA) concluyó en 1998 que las aflatoxinas se encuentran entre las sustancias conocidas con mayor poder mutagénico y carcinógeno (JECFA, 1998).

Interacción con ácidos nucleicos

Los heteroátomos de las bases nitrogenadas de los ácidos nucleicos son muy susceptibles al ataque nucleofílico de los metabolitos de la AFB1, formando enlaces covalentes. Además, la conformación plana del anillo de ciclopentenona fusionado con la lactona de la cumarina, facilita el proceso de intercalado con el ADN (o ARN). Cualquier alteración en la estructura de ácidos nucleicos (ADN y ARN) inhibirá su acción programada y en último lugar, inhibirá la síntesis de proteínas (McLean y Dutton, 1995).

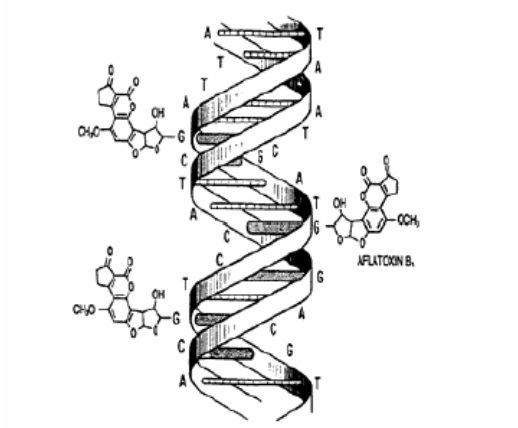


Figura 3: Aducto ADN-AFB1 (Urrego Novoa y Díaz, 2006)

El epóxido de aflatoxina se une covalentemente al nitrógeno 7 de los residuos guanil del ADN (o ARN). Se forma el aducto 8,9-dihydro-8-(N7-guanil)-9-hidroxi-AFB1 (AFB1-N7-Gua) como lesión primaria (McLean y Dutton, 1995), que evoluciona rápidamente a lesiones secundarias que pueden ser: lugares apúricos ("AP sites"), que son bases apúricas o apirimidínicas (en rosa en la

figura 4) o aductos de anillos abiertos de purina, que son las AFB1-formamidopirimidinas (AFB1-FAPY, en verde en la figura 4) (Smela *et al.*, 2002). Estas lesiones son reparadas por mecanismos de escisión, dando lugar a roturas de una hebra o de las dos previos a la reparación (EFSA, 2007). Estudios recientes indican que el compuesto mutagénico es el AFB1-FAPY. Este aducto causa la transversión G>T con mucha mayor frecuencia que la lesión primaria y es un gran bloqueador en la replicación del ADN. En humanos de zonas con gran prevalencia del HBV se han observado transversiones G>T en el codon 249 del gen p53 (Waters *et al.*, 1992; Bedard y Massey, 2006). Los metabolitos de los aductos tras la reparación se pueden encontrar en orina y son un buen indicador de la exposición (EFSA, 2007; Urrego Novoa y Díaz, 2006).

Interacción con proteínas: unión e inhibición enzimática

La AFB1 puede unirse a proteínas en forma de su metabolito AFB1-8,9-dihidroindol, transformándose en un dialdehido y formando bases de Shiff. En el caso de la albúmina, reacciona con el grupo amino primario del residuo lisina y forma el aducto albúmina-aflatoxina (AF-alb). Una fracción de la AFB1 bioactivada es capaz de unirse a proteínas celulares y traslocarse a distintos orgánulos e incluso al núcleo. Un ejemplo de la distinta afinidad del dihidroindol de aflatoxina a proteínas sería kinasa> albúmina> anhidrasa carbónica> RNasa pancreática> histonas. La unión de AFB1 a proteínas funcionales inhibe la función de la proteína, particularmente de las enzimas. Si la síntesis de proteínas no se afectara, las proteínas no funcionales serían reemplazadas por otras sintetizadas *de novo*, y su destrucción sería una forma de detoxificar la AFB1 (McLean y Dutton, 1995), pero la inhibición en la síntesis de proteínas es su mayor efecto metabólico e impide el reemplazo de moléculas fundamentales.

Inducción de estrés oxidativo

Amstad *et al.* en 1984 postularon una nueva vía de acción de la micotoxina. La AFB1 sería capaz de ser genotóxica por una vía indirecta a través de intermedios activos de oxígeno, hiperoxidasas lipícas y pequeños aldehídos. En ese estudio, la inducción del intercambio de cromátidas hermanas en linfocitos se daba a muy bajas concentraciones de aductos AFB1-ADN; por tanto, por ellos mismos no se puede explicar toda la genotoxicidad (Amstad *et al.*, 1984). Lee *et al.* (2005) fueron capaces de provocar estrés oxidativo por AFB1 (10 μM) en la línea celular Hep G2 (células de hepatocarcinoma humano) y detectarlo en forma de especies reactivas de oxígeno (ROS) y 8-OHdG (marcador de daño oxidativo en el ADN). Se ha observado que la AFB1 es capaz de

inducir la formación de 8-OHdG en hígado de rata y pato. Esta lesión en el ADN puede inducir la transversión G>T, lo cual resultaría en una contribución a la carcinogenicidad de la AFB1 (Bedard y Massey, 2006).

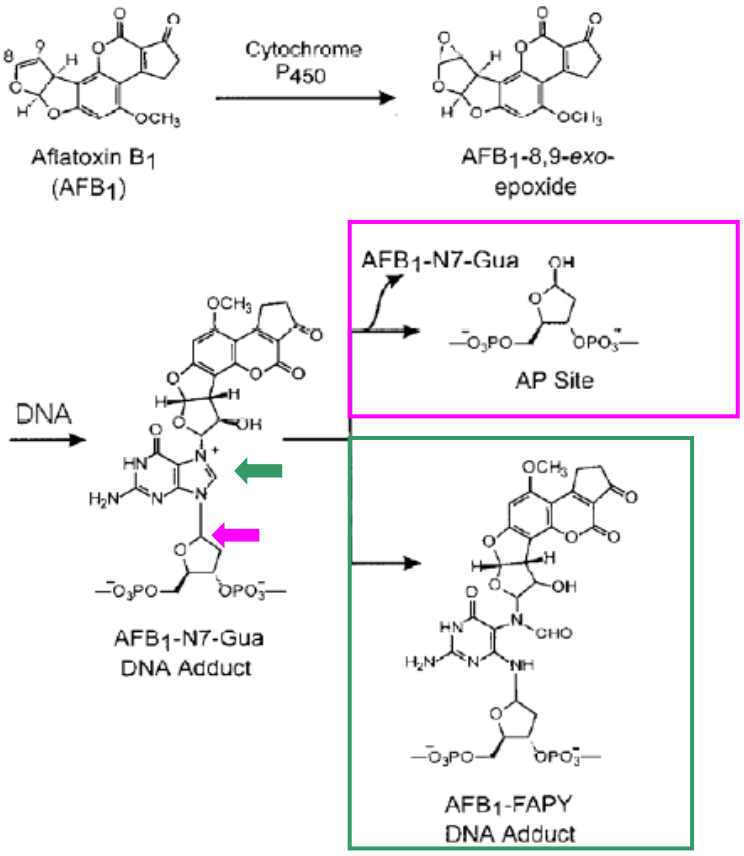
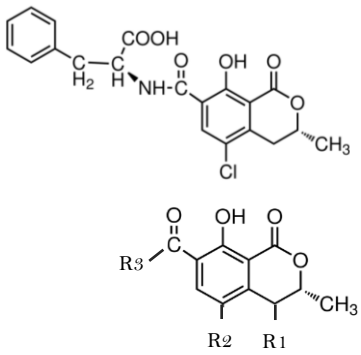


Figura 4: Evolución del aducto AFB1-ADN (Smela et al., 2002)

OCRATOXINA A

Propiedades químicas

Las ocratoxinas son una familia de compuestos estructuralmente relacionados basados en una molécula de isocumarina enlazada a una unidad de L-ß-fenilalanina mediante un enlace amida. Las ocratoxinas naturales más importantes son la OTA y su análogo sin cloro, ocratoxina B (OTB), los correspondientes metil y etil ésteres de la OTA, el derivado 4-hidróxido de la OTA (4(R,S)-OH-OTA) y el ácido carboxílico de la dihidroisocumarina (OTα).



Ocratoxinas	R1	R2	R3
OTA	H	Cl	-NH-CH(COOH)-CH2-fenil
OTB	H	H	-NH-CH(COOH)-CH2-fenil
OTC	H	Cl	-NH-CH(COOH)-C2H-fenil
OTα	H	Cl	-OH
4,hidroxi-OTA	-OH	Cl	- NH-CH(COOH)-C2H-fenil

Figura 5: Estructura de la OTA y sus análogos (Ringot et al., 2006)

La nomenclatura sistemática para la OTA es N-(((3R)-5-cloro-8-hidroxi-3-metil-1-oxo-7-isocromanil) carbonil)-3-fenil-L-alanina. Su fórmula molecular es $C_{20}H_{18}ClNO_6$ y su peso molecular 403,8 g/mol. Es un compuesto cristalino blanco, muy soluble en disolventes polares orgánicos, ligeramente soluble en agua y soluble en soluciones acuosas de carbonatos. Tiene propiedades de ácido débil con valores de pKa entre 4,2-4,4 y 7,0-7,33 (por los grupos carboxilo del resto fenilalanina y el grupo hidroxilo de la parte isocumarina) (Ringot *et al.*, 2006). La OTA es un compuesto estable que no se destruye por procedimientos normales de cocinado, es necesario emplear temperaturas superiores a 250ºC para destruirla. Esta micotoxina es la ocratoxina que se encuentra en mayor concentración en la naturaleza y también es la más tóxica de todas ellas. La OTB suele aparecer en los alimentos junto con la OTA, aunque en menor concentración, y es entre 10 y 20 veces menos tóxica *in vitro* e *in vivo* (O'Brien y Dietrich, 2005; Mally y Dekant, 2005). La OTC (el etil éster de la OTA) es tan tóxico como la OTA (O'Brien y Dietrich, 2005) ya que puede actuar como reservorio y transformarse en OTA dentro del organismo (por hidrólisis del éster en medio

ácido) (Ringot *et al.*, 2006). La OTα (sin el resto fenilalanina) es la menos tóxica de ellas. En general, estudios estructura-actividad sugieren que el resto isocumarina y el grupo lactona están muy relacionados con la toxicidad de las ocratoxinas (Xiao *et al.*, 1996).

Micología

Las ocratoxinas son producidas por los géneros de hongos *Aspergillus* y *Penicillium*. Dentro del género *Aspergillus*, las principales especies ocratoxigénicas son *A. ochraceus, A. niger y A. carbonarius*. Sólo el 50% de las cepas de *A. ochraceus* son capaces de generar la toxina; mientras que aproximadamente el 100% de las de *A. carbonarius* y algo menos del 100% de las *A. niger* tienen esta capacidad. *A. ochraceus* se desarrolla en zonas templadas y cálidas en alimentos como café, cacahuetes y frutos secos; *A. niger* es ubicua y puede aislarse en gran número de sustratos; *A. carbonarius* es menos frecuente; se puede encontrar en vino, uva y uvas pasas. *P. Verrucosum* es la especie ocratoxigénica dentro del género *Penicillium*. Este hongo no se desarrolla a temperaturas superiores a 30ºC, por tanto es típico de zonas templadas o frías (Soriano del Castillo, 2007).

Prevalencia en alimentos, legislación y exposición

La exposición a la OTA se produce principalmente en zonas templadas donde se cultiva trigo, cebada, centeno, arroz, avena, maíz, mijo, café, uvas, cacao, especias, te, dátiles, higos, guisantes, etc. Es relevante su presencia en productos elaborados a partir de materia prima contaminada, como el vino, vinagre, cerveza, chocolate, kétchup, los zumos de frutas, uvas pasas y aceites. La detección en Europa de la presencia de OTA en productos y sangre de cerdo ha demostrado que esta toxina puede pasar de los piensos a los productos de origen animal (FAO/OIEA, 2003). La contribución mayoritaria de OTA en la dieta es a través del consumo de cereales (figura 6).

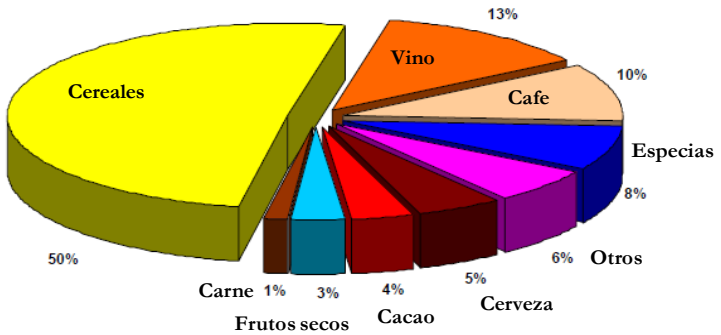


Figura 6: Contribución de los alimentos a la ingesta diaria de OTA en la UE (EC, 2002)

La Unión Europea reguló en 2006 el contenido máximo de OTA en alimentos de consumo humano (Comisón Europea (EC) nº 1881/2006) (EC, 2006). Así, los cereales en general y el café en grano, tostado o molido no deben superar los 5 μg/kg. Los derivados de cereales no pueden superar los 3 μg/kg. Las uvas pasas y el café soluble pueden contener hasta 10 μg/kg. Los vinos y zumos de uva no superarán los 2 μg/kg y los alimentos de consumo infantil no podrán superar los 0,5 μg/kg. En 2010 la Unión Europea reguló también el contenido máximo en especias y regaliz siendo 15 y 20 μg/kg, respectivamente (EC, 2010).

La JEFCA en 1991 estableció una IDT de 16 ng/kg de pc que corresponde a un consumo semanal de 112 ng/kg (JEFCA, 1991). Posteriormente, el Comité Científico de la Comisión Europea sobre alimentación humana valoró los datos científicos sobre la OTA, y, debido a sus posibles efectos cancerígenos, consideró que sería prudente reducir la exposición tanto como fuera posible y en cualquier caso siempre inferior a 5 ng/kg de pc y día, lo que corresponde a 35 ng/kg pc semanales (EC, 1997b). En el informe del proyecto SCOOP Task 3.2.7 en 2002, se presentan los datos de ingesta diaria de OTA en 12 países europeos, siendo, por ejemplo en España, de 1,18 ng/kg de pc (EC, 2002). El consumo medio de OTA en Europa es de unos 45 ng/kg de pc por semana, suponiendo un peso medio de 60 kg, y la mayor exposición sería debida a cereales (58%) (WHO, 2001). El comité de expertos de la JEFCA determinó, en la opinión publicada el 23 de Septiembre de 1994, que la OTA es un potente agente nefrotóxico, un carcinógeno en roedores y que tiene propiedades genotóxicas; por tanto, era necesario determinar una exposición diaria aceptable a esta sustancia. Al crecer la preocupación sobre los efectos potencialmente genotóxicos de la OTA, dicho comité consideró el 17 de septiembre de 1998, reducir su exposición al máximo y en cualquier caso no superar los 5 ng/kg de pc (JECFA, 1998).

Metabolismo

En la mayoría de especies animales, el perfil cinético de la OTA se ha descrito como un modelo de dos compartimentos, pero los datos de acumulación renal sugieren que este modelo es demasiado sencillo y se recomienda un re-análisis de los datos en un modelo multi-compartimental (EFSA, 2006). En humanos, el modelo bicompartimental sugiere una fase rápida de absorción y distribución ($t_{1/2}$ de 20 horas) y una lenta eliminación, con una semivida plasmática de unos 35 días (Studer Rohr *et al.*, 2000). Tras la ingesta oral, es rápidamente absorbida, pasa a circulación sistémica por vía portal y se une a proteínas plasmáticas, en su mayoría albúmina (en un 99,98% en humanos),

lo cual facilita su absorción pasiva y explica en parte la larga vida media en el organismo. Debido a esta alta afinidad por las proteínas, la OTA puede encontrarse en riñón, hígado, grasa y músculo (Kuiper Goodman y Scott, 1989).

La biotransformación de la OTA no ha sido elucidada por completo y su metabolismo sigue generando controversia. Al tener la OTA y algunos de sus metabolitos el resto fenilalanina, se comporta como un análogo de este aminoácido y, por tanto, puede interferir en todas las vías que lo implican (Dirheimer y Creppy, 1991). Las enzimas involucradas en estos procesos son: isoformas del citocromo P450, prostaglandina sintasa (PGSH) y lipooxigenasas (LOX). La vía metabólica mayoritaria es la hidrólisis del enlace peptídico hacia un compuesto mucho menos tóxico como es la OTα (figura 7). Enzimas microsomales humanas (cerdo y rata) son capaces de hidroxilar la OTA (figura 7) a dos epímeros 4(R),4(S)-OH OTA (4(R)-OH OTA en rata y humano y 4(S)-OH OTA en cerdo). Estos compuestos no son tóxicos, debido a su rápida eliminación en comparación con la OTA. Se ha encontrado *in vitro* la presencia de otro derivado hidroxilado: el 10-OH OTA que podría aparecer también *in vivo* (figura 7). El derivado no clorado, la OTB, aparece junto con la OTA en cereales y como metabolito tras la incubación *in vitro* en células de riñón de mono (figura 7). La OTB es 10 veces menos tóxica en pollitos, no tóxica en patitos y ratas (Ringot *et al.*, 2006) y aparentemente no es genotóxica (Knasmuller *et al.*, 2004).

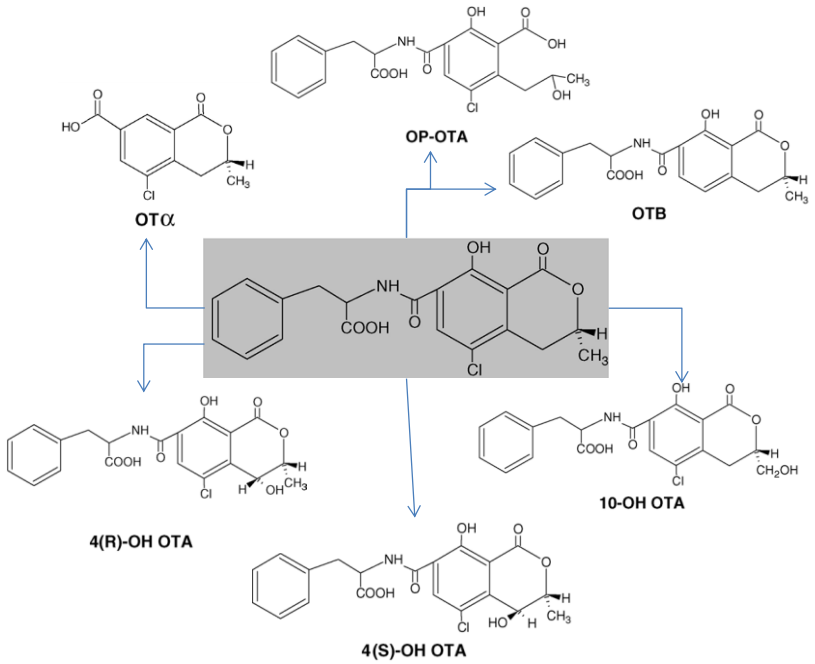


Figura 7: Metabolismo de la OTA

Los estudios cinéticos revelan un lento aclaramiento y, por tanto, una larga vida plasmática en general, aunque existen grandes diferencias entre especies. La excreción es mayoritariamente renal, aunque en roedores prevalece la excreción biliar. La OTA y su metabolito principal, OTα, son excretados por las heces debido a la excreción biliar, que es muy eficaz. Pero la excreción biliar hace que la OTA entre en recirculación enterohepática, y se aprecien segundos picos de distribución en sangre y redistribución por los tejidos. Otra vía secundaria de excreción es la leche materna, vía de exposición muy importante para bebes lactantes (EFSA, 2006).

En la eliminación renal, la filtración glomerular (ultrafiltro pasivo del plasma, dejando paso a solutos e impidiendo el paso de proteínas) es limitada debido a la alta afinidad de la OTA por proteínas plasmáticas. Los transportadores de aniones orgánicos (oat) de las membranas basolaterales introducen la OTA en la célula epitelial y por un transportador similar pasan a las células del túbulo proximal. Los transportadores hOAT1 (predominantes en el riñón) y hOAT3 (presente en hígado y cerebro) se han identificado como mediadores del transporte de la OTA. La OTA sale de la célula a través de la membrana apical del túbulo proximal por difusión facilitada, pero puede ser reabsorbida en todos los segmentos de la nefrona por la presencia de los transportadores oat (como el hOAT4 de la membrana apical del túbulo proximal). Se han sugerido otras vías de reabsorción tubular, como el co-transportador H+-dipeptidasa y la difusión pasiva no iónica (Ringot *et al.*, 2006). Estos procesos de recirculación favorecen el lento aclaramiento de la toxina y la bioacumulación en el riñón (Ringot *et al.*, 2006).

La biotransformación de la OTA en sus metabolitos no explica el largo espectro de efectos tóxicos de la OTA. Se sugiere que se dan reacciones de bioactivación que explican los efectos nefrotóxicos y carcinogénicos.

Toxicidad general

En humanos se conoce un caso de ocratoxicosis aguda debido a la inhalación prolongada de la micotoxina (Di Paolo *et al.*, 1994); sin embargo, las intoxicaciones agudas relevantes aparecen en animales de granja (Hussein y Brasel, 2001). Son más sensibles a la OTA los patos, pollos, pavos y cerdos (DL_{50} 0,5; 3,3; 5,9; 6,0 mg/kg pc, respectivamente) que la rata o el ratón (DL_{50} 20; 46 mg/kg pc) (O'Brien y Dietrich, 2005). Los rumiantes son muy poco sensibles a la OTA, ya que es destruida en sus estómagos (Ringot *et al.*, 2006).

La OTA es principalmente nefrotóxica. En humanos se ha relacionado con la nefropatía endémica de los Balcanes y un aumento en la incidencia de tumores en el tracto urinario, aunque los datos epidemiológicos no son concluyentes (Pfohl Leszkowicz, 2009). El consumo crónico de pienso contaminado con OTA se ha asociado con la nefropatía aviar espontánea (Hamilton *et al.*, 1982) y la nefropatía porcina (Krogh *et al.*, 1979), siendo el cerdo una de las especies más sensibles a la nefrotoxicidad de la OTA. En estudios de toxicidad a dosis repetida, la OTA ha sido nefrotóxica en todas las especies monogástricas testadas, habiéndose observado diferencias significativas entre sexos y especies (Kuiper Goodman y Scott, 1989). Las diferencias entre especies están muy relacionadas con la unión de la OTA a proteínas plasmáticas y su aclaramiento renal (Hagelberg *et al.*, 1989), mientras que las diferencias entre sexos no están elucidadas aún (Vettorazzi *et al.*, 2010; Vettorazzi *et al.*, 2011). Estudios en ratas han confirmado que los túbulos contorneados distales de las nefronas de la franja exterior de la médula externa del riñón son dianas específicas de la OTA (Munro *et al.*, 1974).

La OTA ha demostrado efectos neurotóxicos en ratón (Sava *et al.*, 2006a), rata (Wangikar *et al.*, 2004a) y conejo (Wangikar *et al.*, 2005), y es capaz de bioacumularse en el cerebro en función del tiempo. El mesencéfalo ventral y el cerebelo parecen ser las zonas más sensibles a la OTA (Belmadani *et al.*, 1998). Sava *et al.* (2006b) observaron disminución de dopamina y aumento de estrés oxidativo en diferentes regiones cerebrales de ratones tratados con OTA. Los autores apoyan la idea de que la OTA afecta a la vía nigro-estriada y puede aumentar el riesgo a desarrollar la enfermedad de Parkinson. Otros efectos tóxicos asociados a la OTA son su inmunotoxicidad (Sharma, 1993; Alvarez *et al.*, 2004) y teratogenicidad (Wangikar *et al.*, 2004a; Wangikar *et al.*, 2004b; Wangikar *et al.*, 2005). Según la EFSA, en un estudio en cerdos a 90 días, la mínima dosis de OTA para generar efectos neurotóxicos e inmunológicos es 6 veces la dosis nefrotóxica, y 20 veces para observar efectos teratogénicos (EFSA, 2006).

Genotoxicidad: mecanismos moleculares

La carcinogenicidad de la OTA ha sido evaluada en rata y ratón y se ha observado un aumento en la incidencia de tumores hepatocelulares en ratón, y adenomas y carcinomas renales en ratones y ratas macho (NTP, 1989; Bendele *et al.*, 1985; Castegnaro *et al.*, 1998), aunque la OTA administrada junto con pienso fue significativamente menos carcinogénica que administrada directamente vía sonda gástrica (Mantle *et al.*, 2005). La IARC ha determinado que no existe suficiente evidencia de

carcinogenicidad en humanos, pero sí suficiente evidencia de carcinogenicidad en animales de experimentación; por tanto, clasificó la OTA como posible carcinógeno humano (IARC, 2002). Los mecanismos moleculares de la genotoxicidad de la OTA aún no han sido elucidados.

Se han realizado numerosos estudios para evaluar la mutagenicidad de la OTA obteniéndose resultados contradictorios. Arbillaga *et al.* en 2004 revisaron los resultados más relevantes sobre la capacidad de la OTA para causar mutación génica, reparación de ADN, fragmentación de ADN y alteraciones cromosómicas (Arbillaga *et al.*, 2004). Ni la OTA ni sus metabolitos fueron capaces de generar mutaciones en bacterias, a no ser que en el medio de cultivo hubiera células íntegras (de Groene *et al.*, 1996). Sin embargo, la OTA fue capaz de inducir procesos de reparación de ADN en hepatocitos y otras células diana. Tanto *in vitro* como *in vivo*, existen resultados positivos y negativos sobre la capacidad de generar roturas en la cadena de ADN en forma de intercambio de cromátidas hermanas, alteraciones cromosómicas, formación de micronúcleos y migración de fragmentos en electroforesis en gel (Arbillaga *et al.*, 2004).

Si bien la genotoxicidad de la OTA está demostrada, los mecanismos por los que esta micotoxina daña el ADN no lo están en absoluto. La OTA se ha estudiado desde dos mecanismos genotóxicos contrarios: como genotóxico directo, estudiando la formación de aductos de ADN, o como genotóxico indirecto (epigenético), dañando el ADN por un aumento de estrés oxidativo.

Formación de aductos de ADN

Se han buscado aductos como uniones covalentes entre la OTA o sus metabolitos con ADN de ratón y rata sintetizando patrones del aducto mayoritario de la OTA (O-C8 OTA-3'-dGMP) y buscándolo *in vivo* mediante la técnica de ^{32}P-postlabeling (Pfohl Leszkowicz *et al.*, 1991; Pfohl Leszkowicz *et al.*, 1993; Faucet *et al.*, 2004; Pfohl Leszkowicz y Castegnaro, 2005; Mantle *et al.*, 2010). Sin embargo, estudios posteriores observaron que la técnica ^{32}P-postlabeling no es específica y no se puede asegurar que en los aductos detectados esté presente la molécula de OTA. En muestras de animales tratados con OTA marcada radiactivamente (^{3}H-OTA) (Gautier *et al.*, 2001a) o analizadas por espectroscopía de aceleración de masas (AMS) o cromatografía líquida MS/MS no se han encontrado aductos de OTA (Mally *et al.*, 2004; Mally, 2005; Delatour *et al.*, 2008) y sus autores proponen mecanismos alternativos al daño directo de la OTA o sus metabolitos.

Estrés oxidativo y formación de especies reactivas de oxígeno (ROS)

La toxicidad de la OTA se ha relacionado con el estrés oxidativo aunque no está claro si se debe mayoritariamente a una inducción de ROS o a un descenso en las defensas antioxidantes intracelulares.

Gautier *et al.* (2001B) examinaron *in vivo* la formación de marcadores de estrés oxidativo y encontraron alteraciones, específicamente en riñón, de marcadores de estrés temprano como hemooxigenasa 1 (HO-1) y el marcador de estrés de membrana α-tocoferol, pero no inducción de marcadores de estrés tardío como el aumento de malondialdehido (MDA, producto final de la peroxidación lipídica y marcador de daño renal) o daño en el ADN por formación de 8-oxo-guaninas (8-oxodG). Sin embargo, en estudios *in vitro* se ha confirmado la formación de MDA y aductos 8-oxodG. Las discrepancias entre el *in vitro* el *in vivo* pueden ser debidas a los mecanismos de defensa antioxidante que *in vivo* contrarrestan el ROS formado, pero que no están presentes en los modelos *in vitro*.

Estudios en líneas celulares (Hep G2, Caco 2, MDCK, BME-UV1, HK-2) han mostrado la capacidad de la OTA de inducir ROS, reducir la viabilidad celular e inhibir la síntesis de ADN y proteínas; y cómo algunos antioxidantes (α-tocoferol, retinol, N-acetilcisteína) son capaces de proteger las células (Baldi *et al.*, 2004; Renzulli *et al.*, 2004; Guerra *et al.*, 2005; Costa *et al.*, 2007; Arbillaga *et al.*, 2007). También se ha relacionado la inducción de radicales con daño en el ADN (Arbillaga *et al.*, 2007; Ehrlich *et al.*, 2002; Lebrun y Follmann, 2002; Kamp *et al.*, 2005; Simarro Doorten *et al.*, 2006; Zeljezic *et al.*, 2006).

Dai *et al.* (2002) propusieron dos hipótesis que podrían explicar cómo la OTA es capaz de inducir ROS. Una posibilidad sería (figura 8) que la OTA^{2-} (forma favorecida por el *pH in vivo* (A)) se trasformara en un radical fenólico (figura 8, B) que puede tomar dos rutas. Como se indica, en la ruta C puede oxidar el glutation (GSH) y formar el radical tiilo ($GS^{\bullet}$), que reacciona con otro radical dando un disulfuro de glutation (GSSG), y el radical anión superóxido ($O^{2-\bullet}$). Otra posibilidad (figura 8, D) sería que el radical fenólico participara en una ruta vía radical libre transformándose en una quinona (OTAQ) y generándose $O^{2-\bullet}$ (figura 8, E) en su equilibrio quinona/hidroquinona (OTAQ/OTAHQ). El GSH puede actuar como sustrato y conjugarse con la OTAHQ dando OTAHQ-GS que, en un proceso oxidativo, genera el conjugado cisteinilo (OTAHQ-cisteinil) que puede generar ROS o unirse covalentemente a proteínas (figura 8, F y G). La otra hipótesis postula

que la OTA en su forma fenólica (OTA^-) sea convertida en un catión fenoxonio (figura 8, J) que a su vez se transforme en la OTAQ (figura 8, K) (Ringot *et al.*, 2006; Dai *et al.*, 2002).

Por otro lado, la administración repetida de OTA es capaz de reducir significativamente los niveles de antioxidantes intracelulares como glutatión (GSH), superóxido dismutasa (SOD), catalasa (CAT) o glutatión peroxidasa (GSPx) en hígado y riñón (Meki y Hussein, 2001) y aumentar la peroxidación lipídica (Khan *et al.*, 1989). Estudios estructura-actividad han postulado que el átomo de cloro es esencial para el efecto genotóxico de la OTA, ya que los compuestos clorados que inducen daño en el ADN, sufren antes un proceso de bioactivación a benzoquinonas (Ringot *et al.*, 2006).

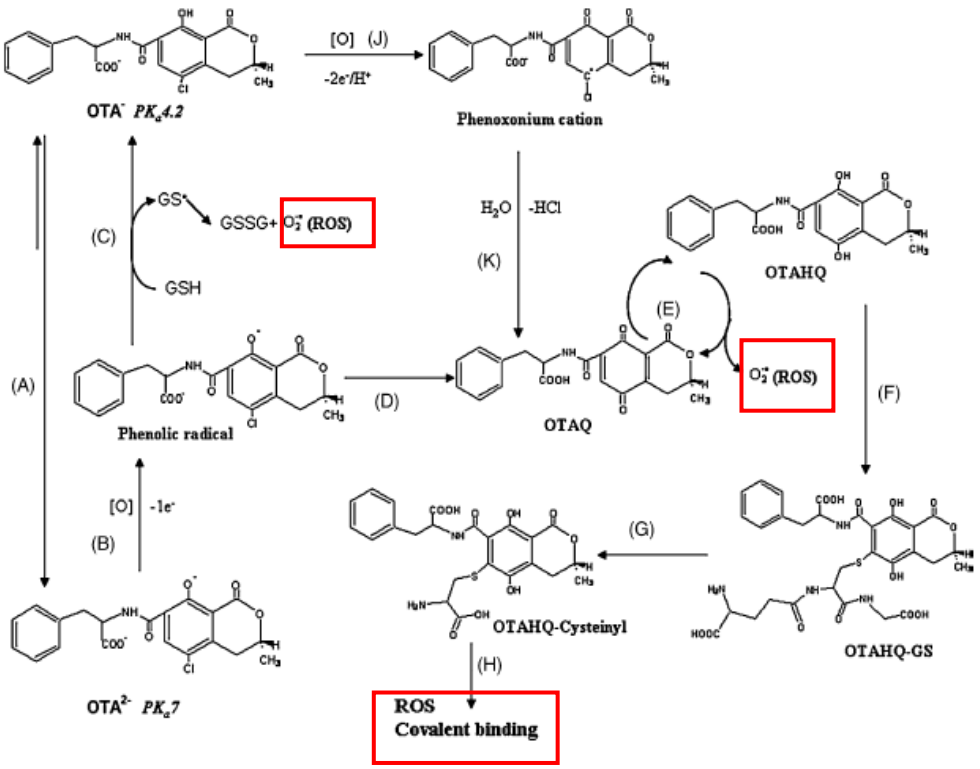


Figura 8: Formación de ROS por la OTA (Ringot et al., 2006)

Estudios de expresión génica han probado que la OTA es capaz de reducir la expresión de genes involucrados en la protección oxidativa intracelular más intensamente en hígado que en riñón (Cavin *et al.*, 2007; Arbillaga *et al.*, 2008); y que aumenta la expresión de la óxido-nitrico sintetasa

inducible (iNOs), enzima responsable de la producción de óxido nítrico (NO). El NO puede reaccionar con el O^{2-}• y generar peroxinitritos que evolucionan a especies reactivas de nitrógeno (RNS) que reaccionan con ADN y proteínas (Marin Kuan *et al.*, 2011).

La información disponible sugiere que la OTA es poco probable que actúe a través de un único mecanismo de acción. La OTA, especialmente en el riñón, genera estrés oxidativo directamente (generación de radicales que alteran los ciclos redox) e indirectamente (disminución de las defensas antioxidantes). Ambos mecanismos pueden interactuar, ya que la reducción de las defensas amplifica el impacto de la producción de radicales libres (Marin Kuan *et al.*, 2011). Por tanto, el mecanismo de acción propuesto como responsable de la carcinogenicidad de la OTA sería una red de interacción de mecanismos epigenéticos, incluyendo la inhibición de la síntesis de proteínas, el estrés oxidativo y la activación de determinadas vías de señalización celular (Marin Kuan *et al.*, 2008).

TOXICIDAD COMBINADA

Aunque la literatura científica ofrece gran cantidad de información sobre el efecto individual de las toxinas, la exposición múltiple y simultánea es un hecho que se ajusta más a la realidad. Por ejemplo, aflatoxinas y fumonisinas, DON (u otros tricotecenos) y ZEA aparecen frecuentemente co-existiendo en el mismo cereal (Ibáñez-Vea *et al.*, 2011; Ibáñez-Vea *et al.*, 2012), en higos (Bircan, 2009), uvas (El Khoury *et al.*, 2008), arroz (Nguyen *et al.*, 2007), o productos elaborados como los cereales de desayuno (Villa y Markaki, 2009). Estudios de exposición conjunta confirman la presencia de biomarcadores de exposición a OTA, AFB1 y AFB2 en niños de Sierra Leona (Jonsyn Ellis, 2001), OTA y aflatoxinas en pacientes con enfermedades renales crónicas en Sri Lanka (Desalegn *et al.*, 2011), o tricotecenos, aflatoxinas y OTA en individuos expuestos a mohos en EEUU tras el huracán Katrina (Hooper *et al.*, 2009).

La combinación de varias micotoxinas en un organismo puede provocar distintos efectos:

- Aditivos: el efecto final es la suma de los efectos individuales.
- Antagónicos: el efecto final es menor que la suma de los efectos individuales.
- Sinérgicos: el efecto final es mayor que la suma de los efectos individuales.

En 2006, McKean *et al.* (2006a y 2006b) presentaron dos trabajos con similar metodología para estudiar los efectos sinérgicos de la AFB1 con la toxina T-2 y la FB1 en modelos *in vitro* e *in vivo*. Emplearon dos líneas celulares: Hep G2 (hepatocarcinoma humano) y BEAS-2B (epiteliales de pulmón humano), ratas F344 y peces *Gambusia affinis*. Con respecto a la citotoxicidad, en Hep G2 la toxicidad conjunta fue aditiva para AFB1+T-2 y ligeramente antagonista para AFB1+FB1 mientras que en BEAS-2B ambas combinaciones fueron sinérgicas. En ratas y peces las combinaciones con AFB1 fueron aditivas tras una dosis. Sin embargo, en ratas Wistar la presencia de FB1 potenció el efecto de la AFB1 en un estudio a dosis repetida (Pozzi *et al.*, 2001)

Creepy *et al.* en 2004 estudiaron la citotoxicidad conjunta entre OTA y FB1 en las líneas celulares Caco-2 (adenocarcinoma colorectal humano) y Vero (epiteliales de riñón de mono cercopiteco verde). Los resultados mostraron un efecto sinérgico entre ambas micotoxinas. La FB1 sola es un tóxico débil, y la OTA un tóxico moderado, mientras que conjuntamente, muestran una toxicidad superior a la suma de sus efectos aislados. Los autores atribuyen el efecto sinérgico a que

ambas generan ROS e impiden la síntesis de proteínas y otras macromoléculas (Creppy *et al.*, 2004). En 2006, Heussner *et al.* estudiaron la citotoxicidad de OTA, OTB, CIT y PAT individualmente, en combinaciones 2 a 2 y todas en conjunto en células renales LLC-PK1 (epiteliales de riñón de cerdo). Individualmente observaron el siguiente órden de toxicidad tras 24 h de exposición: PAT > OTA ≥ OTB > CIT. Sin embargo, en los tratamientos 2 a 2 CIT + OTA > OTB + OTA > PAT + OTA y también CIT + OTB > PAT + OTB > OTA + OTB. Concluyen que existe un efecto sinérgico entre la CIT y la OTA y posiblemente con otras micotoxinas en células renales. Así pues, la toxicidad de éstos compuestos combinados no puede predecirse en base a los resultados de citotoxicidad individual.

Datos *in vivo* mostraron que combinaciones de OTA con CIT pueden tener efectos nefrotóxicos aditivos o sinérgicos en aves, cerdos, ratón y rata (Pohland *et al.*, 1992; Speijers, 2004). Sin embargo, las combinaciones de OTA+T2 y OTA+DON no mostraron interacciones en pollitos y cerdos, respectivamente (Ringot *et al.*, 2006).

Con respecto a combinaciones de AFB1 y OTA existe un número limitado de trabajos hasta la fecha. Ha sido publicado recientemente que AFB1 y OTA muestran una asociación citotóxica ligeramente aditiva en células Vero (epiteliales de riñón de mono cercopiteco verde) y que la mezcla era capaz de aumentar la fragmentación de ADN en comparación con los tratamientos individuales (El Golli-Bennour *et al.*, 2010). Sedmíková *et al.* (2001) estudiaron la mutagenicidad de las dos toxinas individualmente y en combinación con el fin de encontrar sinergias entre ellas. Llegaron a la conclusión de que, si bien la OTA daba resultados negativos en el Test de Ames con *Salmonella typhimurium* (TA98 y TA100), con y sin activación metabólica, la presencia de OTA aumentaba significativamente el efecto mutagénico de la AFB1.

En aves, las AFB1 y OTA tuvieron efecto sinérgico aumentando la mortalidad, pero la OTA inhibió la acumulación lipídica en el hígado que habitualmente genera la AFB1 (Huff y Doerr, 1981; Huff *et al.*, 1983; Huff *et al.*, 1988). En cerdos, no mostraron interacción con respecto a parámetros bioquímicos séricos, pero fueron antagonistas con respecto al daño renal (Ringot *et al.*, 2006). En ratas, la mezcla no mostró interacción con respecto a la mortalidad, ganancia de peso, histología hepática y la mayoría de parámetros séricos (Ringot *et al.*, 2006); mientras que en estudios sobre la teratogenicidad de la mezcla, las lesiones en cerebro, hígado y riñón de fetos fueron menores que cuando se administraban individualmente (Wangikar *et al.*, 2004a; Wangikar *et al.*, 2004b). En gallinas

ponedoras se observaron menores cantidades de AFB1 y OTA en pechuga, hígado y riñón así como en los huevos puestos (Zahoor Ul Hassan *et al.*, 2011).

Los datos existentes muestran que son poco habituales los estudios adecuados para establecer antagonismos, sinergias y efectos aditivos y su interpretación resulta complicada. Como punto de partida, se puede intentar entender la toxicidad combinada de las micotoxinas en función de su mecanismo de acción individual en la célula. Así, en micotoxinas con modos de acción similares se podrían esperar efectos aditivos, o incluso algunas interacciones podrían ser antagonistas (Speijers, 2004). En el caso de la AFB1 y la OTA, los mecanismos de acción son muy dispares, pero ambos comienzan en la biotransformación en el citocromo P450, así que podría darse cualquier tipo de asociación. En la práctica, el resultado cuantitativo o cualitativo obtenido puede ser muy distinto al esperado y, como se ha revisado, AFB1 y OTA pueden dar cualquier tipo de interacción entre ellas *in vitro* e *in vivo*. El resultado parece depender de las especies o del estudio de toxicidad realizado o incluso del tipo de criterio de valoración empleado.

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Chapter 2

Aim, objectives and outline

This work is included in a line of research carried out in the department of Nutrition and Food Sciences, Physiology and Toxicology, which studies aflatoxin B1 and ochratoxin A from the point of view of combined exposure and toxicity.

Until today, many OTA and AFB1 studies have been performed in order to shed some light on the toxicity in humans, animals and cells, but most of them have been carried out in conditions of single exposure to one mycotoxin. However, it has been demonstrated that the majority of basic food staples are contaminated with more than one toxin. Therefore, it seems to be more realistic to study their toxicity in combination rather than one by one.

There is a higher probability of being exposed to low doses of several mycotoxins at the same time throughout life than to a high level of only one mycotoxin; therefore, the effects after chronic exposure are more relevant than the acute ones. Due to the fact that aflatoxin B1 is one of the most potent genotoxic carcinogens and that ochratoxin A is also an indirect genotoxin, it would be very interesting to study the combined genotoxic effects.

The aim of this research project was to explore the combined toxicity of aflatoxin B1 and ochratoxin A in cell systems and *in vivo,* focusing on the genotoxicity power of the combination with comparison to the single effects.

The objectives of this research project were:

1. To study the genotoxicity of AFB1 and OTA, alone and in combination, in human liver cells.

2. To evaluate the protective ability of a polyphenol-enriched cocoa extract against the cytotoxicity and ROS induction of AFB1 and OTA alone and combined.

3. To validate a UHPLC-FLD method for the simultaneous detection and quantification of AFB1 and OTA in order to use it in toxicokinetic and toxicological studies.

4. To approach the toxic and kinetic behavior of the mycotoxins AFB1 and OTA when they are co-administered to Fisher 344 rats.

5. To investigate the genotoxic insult of AFB1, OTA and the combination of both after one oral administration to Fisher 344 rats.

The project is outlined as follows:

Chapter 3: In this chapter, the ability of AFB1, OTA and the combination of both to cause DNA strand breaks and oxidative damage is evaluated with and without external metabolic activation after 3 and 24 h in Hep G2 cells. The comet assay complemented with restriction enzymes is performed. The cytotoxicity of the mycotoxins is evaluated in advance under the same conditions. Moreover, the ROS induction capability after 24 h is also studied.

Chapter 4: A polyphenol-enriched cocoa extract has been obtained from cocoa seeds, and its antioxidant properties against free radicals in cell free systems have been tested. In this work, the cytotoxicity and ROS inner induction of the extract is evaluated in Hep G2 cells; afterwards, its protective properties are evaluated against the cytotoxicity of mycotoxins (alone and combined) and ROS induction.

Chapter 5: In this chapter, the validation of a fast and simple ultra high performance liquid chromatography-fluorescence detector (UHPLC-FLD) method for the simultaneous detection and quantification of AFB1 and OTA in rat plasma, liver and kidney is presented. The objective was to use the same extraction method, calibration curves and chromatographic conditions for both mycotoxins and the three matrices. The validation was performed based on the following criteria: selectivity, linearity, precision and accuracy (within-day and between day variability), limits of detection and quantification, recovery, stability of the samples and robustness.

Chapter 6: A single oral dose of a mixture of AFB1 and OTA was administered orally to male Fisher 344 rats. Biochemistry parameters and histopathological observations are commented. Plasma and tissue levels of mycotoxins are analyzed and compared with published kinetic data of mycotoxins alone.

Chapter 7: The genotoxicity of AFB1, OTA and the combination of both are evaluated *in vivo*. For this purpose, two methods are chosen: the micronucleus test and the comet assay complemented with enzymes. First, the *in vivo* comet assay was set up in liver and kidney in order to detect direct DNA strand breaks and oxidative damage with sufficient sensitivity. Next, one oral administration of the mycotoxins was given to F344 rats and samples were taken after 3 h or 24 h. The micronucleus test was performed on bone marrow while the comet assay was performed on the target organs (liver and kidney). In addition, biochemical parameters, histopathological findings and plasma and tissue levels were taken into account in the toxicity evaluation.

Chapter 8: The most important findings of the previous chapters are discussed in this section.

Chapter 9: The main conclusions of the research project are presented.

Chapter 3

Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells

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Resumen

Las micotoxinas aflatoxina B1 (AFB1) y ocratoxina A (OTA) pueden aparecer juntas en un mismo alimentos y si bien actúan por mecanismos diferentes, ambas son consideradas genotoxinas. El objetivo de este trabajo es caracterizar la genotoxicidad combinada de ambas toxinas *in vitro* en la línea celular Hep G2. Para ello, la citotoxicidad se estudió en tratamientos aislados y en combinación con el fin de establecer los rangos de dosis a aplicar en los estudios de genotoxicidad. La citotoxicidad conjunta de AFB1+OTA durante 24 h mostró efectos aditivos. La genotoxicidad se evaluó en la línea Hep G2 mediante el ensayo del cometa complementado con las enzimas de restricción endo III y FPG. La inducción de especies reactivas de oxígeno también fue evaluada en tratamientos aislados y combinados. La AFB1 fue genotóxica tras 3 h con activación metabólica (mezcla S9) y tras 24 h sin activación metabólica. La presencia de OTA disminuyó significativamente el daño en el DNA provocado por la AFB1, no solo en las roturas directas y lugares apurínicos sino también en los lugares sensibles a la FPG. Esta aparente contradicción entre los efectos citotóxicos aditivos y los efectos genotóxicos antagonistas puede deberse a que ambas toxinas compiten por los mismos CYPs, generando más ROS pero menos aductos de AFB1.

Abstract

Mycotoxins aflatoxin B1 (AFB1) and ochratoxin A (OTA) can be present together in food commodities. These food contaminants are considered to be genotoxins, acting by different mechanisms. The aim of this work was to characterize combined genotoxic *in vitro* effects of both mycotoxins in Hep G2 cells. For this purpose, cytotoxicity was first determined in isolated and combined treatments in order to determine the dose range of genotoxicity studies. Co-exposure of cells to AFB1+OTA for 24 h resulted in additive effects. Genotoxicity was determined in Hep G2 cells by the modified comet assay with restriction enzymes (endo III and FPG). Significant reactive oxygen species formation was detected in both single and combined treatments. AFB1 was genotoxic after 3 h with external metabolic activation (S9 mix) and after 24 h without metabolic activation. Co-exposure to OTA significantly decreased DNA damage induced by AFB1, not only in breaks and apurinic sites but also in FPG-sensitive sites. The apparent contradiction between additive cytotoxic effects and antagonic genotoxic effects may be explained if AFB1 and OTA compete for the same CYPs, yielding more ROS but less AFB1 adducts.

Introduction

Mycotoxins are secondary metabolites produced by different fungal species that can contaminate agricultural commodities in fields, during harvest and/or in storage, and can reach human beings through contaminated food. Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are some of the most relevant mycotoxins due to their toxic effects and demonstrated human exposure. The European Union has established maximum permitted levels for both mycotoxins (EC, 2002; EC, 2006).

The International Agency for Research on Cancer (IARC) classified AFB1 as class 1 (human carcinogen) and OTA as class 2B (possible human carcinogen) (IARC, 1987; IARC, 1993; IARC, 2002). AFB1 is a genotoxic hepatocarcinogenic compound which may also cause tumors in other organs, such as colon and kidney (EFSA, 2007). It is bioactivated in liver by cytochrome P450 and its epoxide metabolite attacks DNA forming adducts (McLean and Dutton, 1995) that might evolve to secondary injuries such as apurinic sites (AP) or imidazole AFB1 formamidopyrimidine opened rings (AFB1-FAPY) (Bedard and Massey, 2006). The metabolite AFB1-FAPY induces G-T transversion, and is a good indicator of AFB1 exposure in urine (Urrego Novoa and Díaz, 2006). OTA is a potent nephrocarcinogenic compound in rodents but despite the controversy, it is considered to be an indirect genotoxic agent (Arbillaga *et al.*, 2004; Arbillaga *et al.*, 2007; Mally *et al.*, 2004; Mally *et al.*, 2005; Mally and Dekant, 2005; Turesky, 2005). OTA mechanisms of action are not clearly determined but apparently, OTA may disrupt phenylalanine metabolism, reduce gluconeogenesis, and induce apoptosis via protein/DNA synthesis inhibition. In addition, the OTA capability to generate reactive oxygen species (ROS) may explain the lipid, protein and DNA damage (Ringot *et al.*, 2006). Moreover, OTA is nephrotoxic, hepatotoxic, teratogenic and immunotoxic (EFSA, 2006) and recent studies are relating it to neurodegenerative diseases such as Parkinson and Alzheimer (Sava *et al.*, 2006a; Sava *et al.*, 2006b; Sava *et al.*, 2007).

Human population is probably exposed to multiple mycotoxins because human diet is generally varied and because the same food might be contaminated by several mycotoxins. Multi-exposure may lead to additive, synergistic or antagonistic effects; however, there are few studies regarding possible mycotoxins interactions. Different mycotoxins could be selected for this purpose. The selection of AFB1 and OTA is based on the fact that both of them are genotoxic, but their mechanism of action is very different so some association might be expected. Multi-evaluation with OTA, ochratoxin C (OTC), citrinin (CIT) and patulin (PAT) reported that even though CIT was the

least toxic of all of these toxins, it was the most interactive compound, and only CIT+OTA act with synergism (Heussner *et al.*, 2006). There were synergic effects between AFB1+T-2 toxin (T-2) and AFB1+fumonisin B1 (FB1) in BEAS-2B, a human bronchus epithelial cell line, while there was only an additive effect in AFB1+T-2 mixtures and a slight antagonism between AFB1-FB1 in Hep G2, human hepatocarcinoma cell line (McKean *et al.*, 2006a; McKean *et al.*, 2006b). OTA and FB1 were also tested together in Caco-2 (human colorectal adenocarcinoma) and Vero (green monkey renal) cell lines. The couple yielded a synergic behavior, possibly due to their ROS production capability (Creppy *et al.*, 2004). With regard to AFB1 and OTA, AFB1 was found to be mutagenic with metabolic activation and OTA was not mutagenic in the Ames test, even though the OTA increased the mutagenicity of AFB1 (Sedmíková *et al.*, 2001); and the mixture not only showed cytotoxic additive effects but also a slight increase in DNA fragmentation as compared to mycotoxins taken separately in Vero cells (El Golli-Bennour *et al.*, 2010). Therefore, there is a real potential hazard in simultaneous occurrence of AFB1 and OTA.

Due to the aforementioned, and according to the European Commission Recommendation of 17 August 2006 L234/35, where the necessity to improve new methods for detecting, quantifying and evaluating mycotoxins joint actions is highlighted, the objective of this work was to evaluate AFB1 and OTA genotoxicity, alone and in combination, in Hep G2 cells. For this purpose, cytotoxicity was first tested; then, genotoxicity was evaluated by the modified comet assay using restriction enzymes endonuclease III (endo III) and formamidopyrimidine DNA glycosylase (FPG).

Materials and methods

Chemicals

Aflatoxin B1 and OTA were obtained from Sigma-Aldrich (Steinheim, Germany). Ochratoxin A was dissolved in 0.1 M $HNaCO_3$ (pH 7.4) (Riedel-deHaën, Seelze, Germany) to an initial concentration of 2.476 mM and AFB1 was dissolved in DMSO (Panreac Quimica SAU, Barcelona, Spain) to a initial concentration of 15.15 mM. All solutions were aliquoted and maintained at -20ºC until use. Aflatoxin B1 was kept in the dark to avoid degradation. Moltox post-mitochondrial supernatant (S9 rat fraction) was purchased from Trinova Biochem (Gieβen, Germany) and stored at -135ºC until use. The S9 mix was prepared by adding the following ingredients: NADP (1M, Sigma Aldrich), glucose-6-Phosphate (1 M, Sigma Aldrich), KCl (1.65 M, Merck), $MgCl_2$ x 2 H_2O (0.4 M, Merck), NaH_2PO_4 x H_2O (0.2 M, Merck) and Na_2HPO_4 (0.2 M Sigma Aldrich).

Cell cultures

Hep G2 (hepatocellular carcinoma epithelial cell line) (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). It was kept in the laboratory satisfying the requirements for Good Laboratory Practice conditions. A master bank was created on arrival and a working bank was created to perform the assays of this study, controlling the passing number, which never surpassed 20.

Cells were cultured in monolayer in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Prat de Llobregat, Barcelona, Spain) supplemented with 10% of fetal calf serum (FCS, Gibco) and 1% of antibiotics (10,000 U/mL penicillin and 10,000 μg/mL streptomycin, Gibco). Cells were grown at 37ºC and 5% CO_2 in a humidified atmosphere.

MTT assay

Cytotoxicity was assessed by the MTT assay which measures the reduction of MTT (3-(4,5-methyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) to formazan by the mitochondrial enzyme succinate dehydrogenase (Mosmann, 1983). Cells were seeded on 96-well plates. After 48 h of incubation, they were treated with AFB1 and OTA, alone and combined. Each concentration was tested in 6 independent wells. Mycotoxins were added to serum-free medium in order to avoid serum binding. Exposure times of all treatments were 3 h (with and without metabolic activation) and 24 h. The final concentration of the metabolic activation S9 mix was 10% and was prepared and added to the cultures together with the mycotoxins and maintained during 3 h. After the treatments cells were washed, and MTT (5 mg/mL) was added to fresh culture medium to each well and incubated another 2 hours. The supernatant was removed, and the insoluble formazan crystals were dissolved with 100 μL of DMSO. The absorbance was measured at 540 nm using a spectrophotometer reader (Titertek Multiskan® MCC/340 MK II). Results were expressed as the percentage of viability (%) with respect to the control (solvent treated cells) according to the following formula: [(absorbance treated cells - absorbance blank)/ (absorbance control cells – absorbance blank)] x 100. The half inhibitory concentration (IC_{50}) was estimated as the measurement of the cytotoxic potency of single and combined treatments.

Comet assay

After single or combined treatments with mycotoxins during 3 h (with and without metabolic activation) or 24 h, cells were washed and detached. In order to monitor the ongoing process of the assay, a negative control (cells treated with solvent) and a positive control (cells treated with a solution of 100 μM H_2O_2) were included. The comet assay was applied as previously described by Singh *et al.* and Collins *et al.* (Singh et al 1988; Collins *et al.*, 1997; Collins and Dusinska, 2002; Collins *et al.*, 2001) with several modifications. The comet assay was performed to detect DNA strand breaks, alkali labile sites (AP), and oxidized DNA bases. The DNA strand breaks were detected with a simple version of the comet assay, but oxidized DNA bases were detected by incubating DNA with bacterial restriction enzymes, which permit detecting specific lesions. Two different enzymes were used in these experiments: endo III, whose substrates are oxidized pyrimidines; and FPG, which is known for recognizing the common oxidized purine 8-oxoGua and ring-opened purines, or formamidopyrimidines (FAPY) (Collins *et al.*, 2008).

Cells were resuspended in PBS at a concentration of 1x106 cells/mL. Thirty microliters of this suspension were mixed with 140 μL of 1% low melting point agarose; two drops of 70 μL of this mixture were placed on a microscope slide. A cover slip was put on top of each drop and the gels were allowed to set for 5 min at 4ºC. Once the gels had become solid, the cover slip was removed and the slides were dipped into lysis solution at 4ºC (2.5 M NaCl, 0.1 M Na_4- EDTA • 2 H_2O, 0.01 M Trizma-BASE, pH 10.5, TRITON X-100 1%). Four different slides, numbered from 1 to 4, were prepared for each condition. Number 1, for observing DNA breaks and AP sites, and numbers 2, 3 and 4 for obtaining information regarding the presence of oxidized DNA bases using endo III and FPG post-treatments.

During at least 1 h, samples were maintained in the lysis solution at 4ºC. The positive control slide cells was dipped into H_2O_2 (100 μM in PBS) solution for 5 min at 4ºC, then washed with cold PBS and introduced into a lysis solution in a separate jar for at least 1h.

Slides 2, 3 and 4 were washed 3 times (5 min each time) with the enzyme buffer (0.04 M HEPES, 0.1 M KCl, 0.0005 M Na_4- EDTA x 2 H_2O, 0.2 mg/mL BSA, pH 8). Then gels were digested with buffer (slide 2), endo III (slide 3) or FPG (slide 4), by adding 50 μL of the corresponding solution to each gel and placing a cover slip on top, for 30 min at 37ºC in a humid chamber. All nucleoids were denatured in a high-pH buffer (0.3 M NaOH, 0.001 M Na_4-EDTA x

2 H_2O) for 40 min at 4ºC. Finally, electrophoresis was carried out at approximately 1 V/cm (Brunborg, 2008) for 30 min and the DNA was gently re-neutralized in PBS during 10 min and washed in H_2O for another 10 min.

Nucleoids were stained by adding a drop of 20 μL of DAPI (1 μg/mL) (Sigma-Aldrich) on each gel and comets were analyzed in a fluorescence microscopy (Eclipse 50 i NIKON). A total of 100 comets were scored and visually classified into five classes (0-4), according to their head and tail intensity (Collins, 2004). Class 0 means no DNA damage or undetectable tails, and class 4 means maximum damage. The total score was calculated using the following equation: (% of cells in class 0 x 0) + (% of cells in class 1 x 1) + (% of cells in class 2 x 2) + (% of cells in class 3 x 3) + (% of cells in class 4 x 4). Consequently, the total comet score was within 0-400 arbitrary units (AU). Arbitrary units from slide 1 represent the DNA strand breaks and AP sites caused by the treatment. Net endo III and FPG-sensitive sites were calculated by subtracting the AU of slide 2 from the AU of the enzymes-treated slides (3 and 4 respectively).

Dichlorofluorescein assay

ROS were determined by using fluorescent probe DCFH-DA (2',7'-dichlorfluorescein-diacetate), as Wang and Joseph described (Wang and Joseph, 1999). DCFH-DA crosses the membranes and is enzymatically hydrolyzed by intracellular esterases in order to form nonfluorescent DCFH (dichlorofluorescein). The latter is rapidly oxidized to form highly fluorescent DCF (dichlorofluorescein) in the presence of ROS. The DCF fluorescence intensity parallels the amount of intracellular ROS. Hep G2 cells were incubated for 30 min at 37ºC in serum-free and phenol red lacking DMEM medium containing 100 mM DCFH-DA. Next, cells were washed once with PBS and exposed to single or combined treatments with the mycotoxins during 24 h in serum-free and phenol red lacking DMEM medium. Each concentration was tested in 6 independent wells. ROS were measured after 24 h using a Fluoroskan Ascent microplate fluorometer (Thermo Labsystems) at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. Afterwards, an MTT assay was performed in order to obtain the exact percentage of cell survival in each well. The value of fluorescence was divided by the percentage of cell survival at each concentration in order to obtain the relative fluorescence (RF), which is then independent of the cell viability. Therefore, the relative fluorescence of the negative control would be 1, and values over 1 suggest an increase of intracellular ROS.

Statistical analysis

Statistical analysis was performed by using SPSS for Windows. Three independent experiments were performed in order to evaluate cytotoxicity and genotoxicity; four independent experiments were carried out in the case of the ROS assay. Data is presented by descriptive analysis [mean±standard deviation (SD) of the replicated experiments]. Comparisons were performed by nonparametric Kruskal-Wallis H-test and Mann-Whitney U-test. $P \leq 0.05$ probability was accepted as the level of significance.

Results

Cytotoxicity

Mycotoxins cytotoxicity was measured in Hep G2 cells at two different exposure times: a short exposure time of 3 h, with and without S9 mix; and a long exposure time of 24 h. First they were studied separately; then in combined treatments.

After 3 h of exposure, there was no evidence of cytotoxicity for AFB1 (1.56-150 μM) and no apparent toxicity for OTA (50-800 μM). The presence of an external metabolizing enzyme system did not show any variation in cell survival (data not shown). The IC_{50} values for AFB1 were over 150 μM and over 800 μM for OTA. After 24 h of treatment, AFB1 was more toxic (IC_{50}= 100 μM) than OTA (IC_{50}= 360 μM) for Hep G2 cells. See table 1 and figure 1.

In order to study the effects of mycotoxins acting together, 100 μM or 150 μM of AFB1 were added to five concentrations of OTA (3 h: 50-800 μM, 24 h: 1-200 μM). In the short treatment of 3 h, no significant cytotoxicity was observed at any of the concentrations of OTA with 100 μM or 150 μM of AFB1. At 24 h of treatment, the mixtures were more cytotoxic than the mycotoxins alone (figure 1), thus decreasing the IC_{50} values (table 1).

Table 1: IC_{50} values in Hep G2 cell line after 24 h of single or combined treatment.

	IC50 μM
	HepG-2
AFB1	100
OTA	360
OTA+100 μM AFB1	100
OTA+150 μM AFB1	200

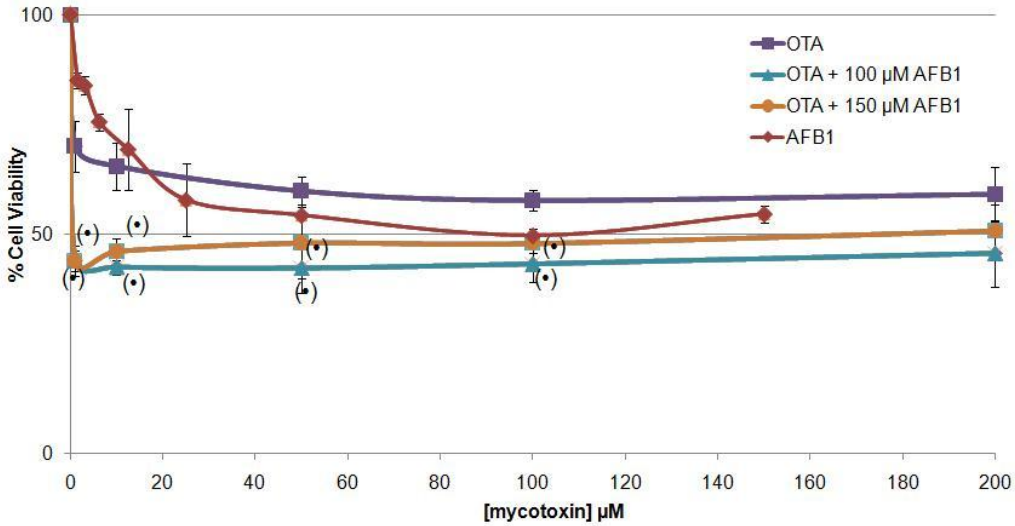

Figure 1: Viability curves of Hep G2 after 24 h of incubation with OTA and AFB1 alone and in combination, obtained with the MTT assay. In order to observe the significant effect of the mycotoxins together, each combination was compared with the single treatment of OTA (• $p \leq 0.05$) or 100 and 150 μM of AFB1 (no signification). The mean and SD of three experiments are shown.

Genotoxicity

At 3 h without S9 rat mix, AFB1 did not induce DNA strand breaks or AP sites (figure 2A), and no significant damage was found after the enzyme treatment (figure 2B). In contrast, when metabolic activation was used, a significant dose-response relationship was evident in direct DNA strand breaks starting at 30 μM (figure 2C). Moreover, significant increase in DNA damage was detected by FPG at the same concentration (figure 2D). After 24 h of treatment with AFB1, a dose-response effect was observed with significant DNA induced strand breaks (figure 2E). In addition, at 6 μM, a clear significant induction of FPG sites was shown that did not appear at 3 h (figure 2F). No

significant variations of the DNA breaks were detected after the digestion with endo III in any of the treatments with AFB1 (figure 2B, 2D, 2F).

Ochratoxin A (50-800 μM) did not induce DNA breaks after 3 h or 24 h of exposure to Hep G2 cells. The enzymatic digestion of the nuclei did not reveal any oxidative damage in the range tested (data not shown).

Combined treatments were only carried out at 24 h because a very good dose-response relationship was observed in single treatments at this time point; in addition, FPG was able to detect significant DNA breaks at lower concentrations of AFB1 at 24 h rather than at 3 h with S9 mix. For this purpose, different concentrations of AFB1 (1.2-150 μM) were tested with 50 μM of OTA. Both combined treatments showed a good dose response behavior in DNA strand breaks and AP sites (figure 3A). According to the enzyme digestion of the nuclei, no increase in DNA damage has been observed when endo III was used (figure 3B) whereas significant increases in DNA breaks were detected by FPG following a dose-response effect (figure 3C). The comparisons between the combined and the single exposures at AFB1 yielded a significant decrease of DNA strand breaks and AP sites (figure 3A). The detected damage in FPG treated nuclei was also less at low AFB1 concentrations (1.2 - 30 μM) (figure 3C).

Induction of intracellular ROS

The induction of intracellular reactive oxygen species (ROS) was studied in Hep G2 cells after 24 h of exposure to AFB1 (1.2-150 μM), OTA (50-200 μM), and the mixture of AFB1 with 50 μM of OTA. There was a dose-dependent induction of ROS with a single treatment of AFB1 (figure 4A). OTA treated cells suffered an increase in their ROS up to 100 μM (1.5 times control level) and then the levels remained stable (figure 4B). Combined treatments induced greater amounts of ROS in comparison to the control, and the differences were statistically significant. There was an increase in ROS when the combined treatment was used with respect to the single treatment. This increase was not significant in any case.

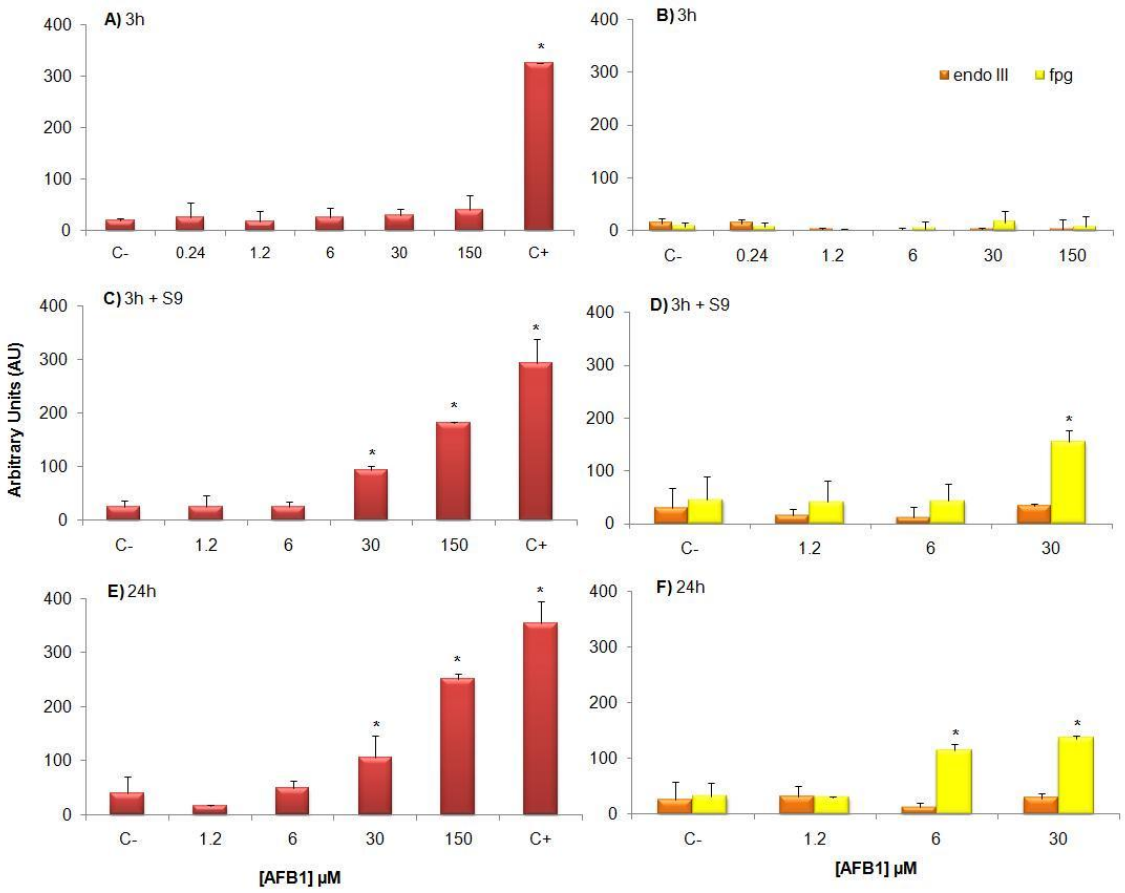

Figure 2: Genotoxic effects of AFB1 after 3 h (A and B), 3 h with 2.5% S9 rat mix (C and D) and 24 h of treatment (E and F) measured with the comet assay. DNA damage was measured in arbitrary units (0-400). DNA strand breaks and AP sites are plotted in A, C and E. Oxidative damage detected by post-digestion with the enzymes is represented as net endo III (orange) and net FPG-sensitive sites (yellow) in B, D and F. In order to observe the significant effects of treatments, each concentration was compared with nontreated cells (C-) ($p \leq 0.05$). The mean and SD of three experiments are shown.*

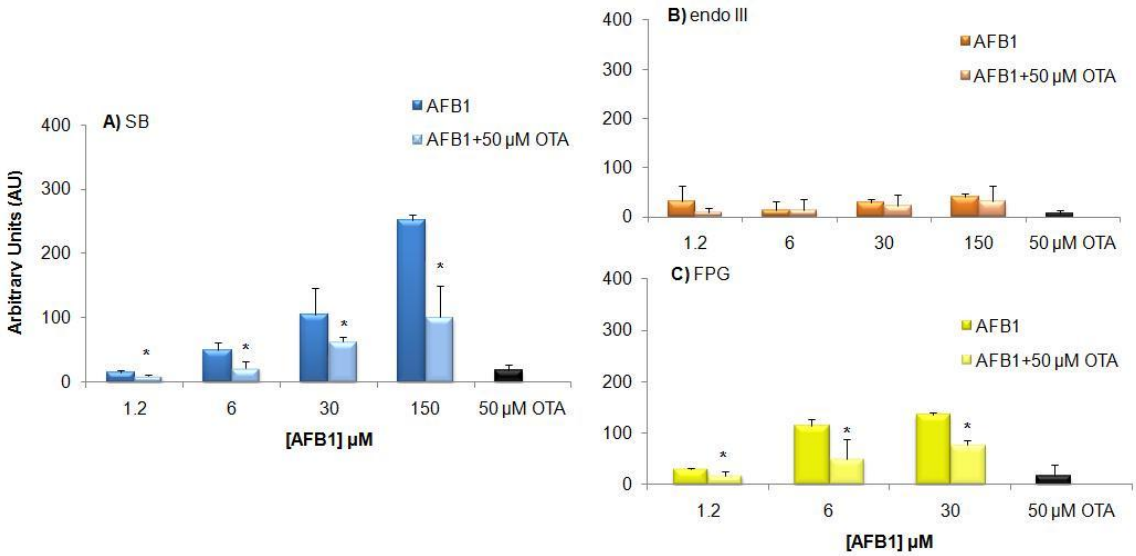

Figure 3: Comparative plots of DNA damage after exposing Hep G2 cells to AFB1 and AFB1+ 50 µM OTA for 24 h measured with the comet assay. DNA damage was measured in arbitrary units (0-400). DNA damage was measured as DNA strand breaks and AP sites (A) or oxidative damage, expressed as net endo III (B) or FPG (C) sensitive sites. In order to observe significant differences among treatments, AFB1+OTA combinations were compared with single exposures to AFB1 (p≤0.05). The mean and SD of three experiments are shown.*

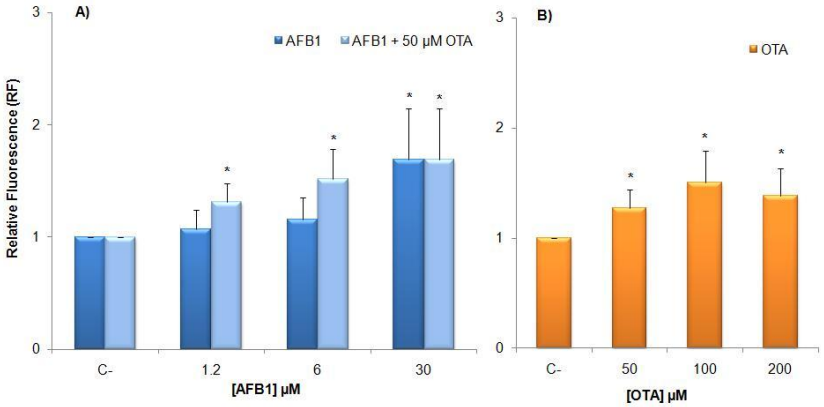

Figure 4: Intracellular ROS of Hep G2 cells treated with AFB1 (A in blue), OTA (B), and mixtures of AFB1+ 50 µM OTA (A in light blue) for 24 h. ROS levels were expressed as fluorescence per percentage of survival. Results were compared with their negative control (p≤0.05). The mean and SD of four experiments are shown.*

Discussion

According to the literature, more than one mycotoxin may occur together in the same grain and furthermore, the manufacturing process mixes together different raw materials, yielding totally new matrixes with a new risk profile (Binder *et al.*, 2007). Until today, many OTA and AFB1 studies have been performed in order to shed some light on the toxicity in humans, animals and cells, but most of them have been carried out with conditions of single exposure to one or another mycotoxin. However, there is a higher probability of being exposed to low doses of several mycotoxins at the same time throughout life than to a high level of only one mycotoxin. Therefore, it seems important to explore their behavior when they coexist inside the same cell.

With the aim of studying the possible genotoxic effects of the mycotoxins, alone and in combination, the comet assay was carried out in Hep G2 cell line. This cell line has been proposed as a good experimental model for detecting mutagenicity and anti-mutagenicity (Knasmuller *et al.*, 1998) and for studying metabolism-induced drug hepatotoxicity (Otto *et al.*, 2008). Hep G2 cells appears to be adequate for performing assays with substances that need metabolization because a certain amount of phase I and phase II metabolizing enzyme activity is apparently maintained (Knasmuller *et al.*, 1998). Moreover, one of the conclusions of the 5th International Workshop on Genotoxicity Testing (IWGT) was that human p53-competent cells, such as Hep G2, may be a good model for *in vitro* mutagenicity assays in mammalian cells –*in vitro* micronucleus or aberration tests- in order to reduce non-relevant positive results for carcinogenicity prediction (Pfuhler *et al.*, 2011). In addition, this cell line has been proven to be useful for detecting genotoxicity with the comet assay after long time treatments with promutagens (Uhl *et al.*, 1999).

Genotoxicity studies were preceded by cytotoxicity studies at short and long time treatments, in order to establish the adequate dose range to be tested in genotoxicity studies. The comet assay has still not been validated; there are no recommended guidelines. For this reason, recommendations regarding mutagenicity assays on mammalian cells have been taken into account (OECD, 1997; OECD, 2010). The comet assay was performed at a short time of 3 h with and without external metabolic competence, to avoid cell reparation of the possible DNA damage as guidelines suggest, but also at 24 h to take advantage of the inner metabolic competence of Hep G2. For AFB1, the highest concentration of 150 μM was in the limit of solubility, because evident signs of precipitation of the compound were observed under the microscope. For OTA, the highest concentration of

800 μM was selected because it was close to 50% of viability, approximately 60%, and also because a very flat dose-response curve was obtained in Hep G2 cells (figure 1).

In combined 24 h treatments, the cytotoxicity observed was more pronounced than in single treatments, and the results are concordant with an additive effect due to the fact that the remaining surviving cells after combined treatment corresponded to the theoretically surviving cells after separate treatments with OTA and AFB1 in the same well. This effect coincide with those reported in Vero cells by El Golli-Bennour *et al.* in 2010 and it suggests that either the mycotoxins do not share cell pathways in their toxic action, or if they share some step, it is expected that they would at least have additive effects (Speijers, 2004).

AFB1 short treatments in the presence of external metabolic activation and long ones without metabolic activation gave positive results. The comet assay was able to detect the different types of DNA damage caused by AFB1 after its bioactivation, as previously described (Bedard and Massey, 2006). A dose-dependent response was observed in the standard comet assay (figure 2 C and E). After AFB1 bioactivation, the reaction of AFB1-exo-8,9-epoxide with guanine gives the adduct AFB1-N7-Gua (figure 5), which is very labile due to the presence of a positive charge on the imidazole ring (Bedard and Massey, 2006). The detected lesions are probably due to the loss of adduct with guanine, which leads to the formation of an AP site or a break in the repairing process (figure 5). Another possible reaction is the base-catalyzed hydrolysis where the imidazole ring opens, forming a stable AFB1-FAPY (figure 5). AFB1-DNA adducts are believed to be repaired by the nucleotide excision repair (NER), but in mammalian systems, AFB1-FAPY is much more resistant to loss by spontaneous or repair processes than the initial AFB1-N7- Gua adduct because the former is less distortive to DNA architecture (Smela *et al.*, 2002). Therefore, the results obtained most likely represent both secondary lesions.

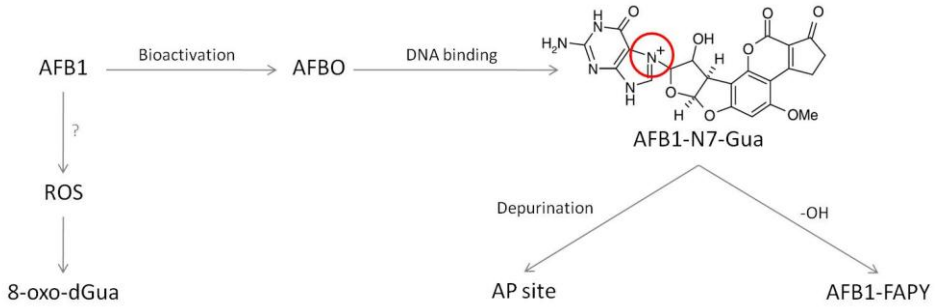

Figure 5: Scheme of the mechanism of action of the DNA damage induced by the AFB1.

It has been described that FPG detects the typical oxidative biomarker, 8-oxodG, and the open ring formamidopyrimidines (FAPY) (Collins *et al.*, 2008). A significant increase in DNA damage was found when nuclei were treated with FPG, and no oxidized purines were detected with endo III. Bedard and Massey (2006) suggested that, as a secondary route, AFB1 may yield ROS forms that react with guanine residues, forming 8-oxodG. In order to elucidate if the significant increase of net FPG-sensitive sites was only due to AFB1-FAPY forms or if oxidative damage was also involved, the dichlorofluorescein assay was performed after 24 h when the positive oxidative damage was clearly observed. A low, but significant, increase in intracellular ROS was detected; therefore, it is presumed that oxidative secondary route may play a role in AFB1 genotoxic action.

No genotoxic effects were observed in Hep G2 cells after 3 h or 24 h treatment with OTA in the concentration range of 50-800 μM, with the latter being clearly cytotoxic after 24 h. These results do not coincide with those reported by other authors, who have found a tail length increase in the same cell line after 24 h, using a lower range (up to 62 μM) (Ehrlich *et al.*, 2002). In human CYP expressing cells, a slight significant increase in tail moment was observed only at the highest cytotoxic concentration used (200 μM), after 8 h of exposure (Simarro Doorten *et al.*, 2006). Arbillaga *et al.* (2007) found a genotoxic effect in HK-2 cells after 6h (not after 3 h) with the comet assay but only at cytotoxic concentrations, and this effect was correlated with an increase in intracellular ROS. Clear increases were found in intracellular ROS levels in the range tested at 24 h, and these results coincide with those published in the same experimental conditions; moreover, the author correlates ROS increases with a mitochondrial-dependant apoptotic process and an upregulation of Hsp70 genes (several authors have shown an evident correlation between oxidative stress generation and Hsp70 induction (Hassen *et al.*, 2005; El Golli *et al.*, 2006)). Conversely, the authors found that the increase of ROS was very low compared to H_2O_2 and lower yet when compared to other cell lines (Arbillaga *et al.*, 2007; Costa *et al.*, 2007), so perhaps the OTA ROS oxidation in DNA was low enough to be undetectable for FPG with the comet assay. A strong hypothesis regarding how OTA produces ROS is that the OTA molecule reacts with glutathione (GSH) or forms OTA-derived quinines (OTA-Q and OTA-HQ), yielding ROS acting forms as secondary products (Ringot *et al.*, 2006). As Hep G2 cells retain some phase I and phase II metabolizing enzymes, they might be able to detoxify OTA, reducing the molecules available to react with GSH, Q or HQ, and consequently, reducing ROS formation.

With respect to the genotoxic combined effects of these two toxins, the results that have been obtained were unexpected. Combined treatments showed a significant decrease in DNA damage, not

only in breaks and AP sites but also in FPG-sensitive sites, when compared to the AFB1 treatment. On the other hand, ROS increased when mycotoxins coexist inside cells. A possible hypothesis for these facts may be that AFB1 and OTA compete for the same CYP enzymes that represent a bioactivation route for AFB1. If OTA had much more affinity for the CYPs involved, it would displace other molecules until OTA would be consumed and consequently, after 24 h, there would be less AFB1 bioactivated molecules (AFB1-epoxide) to attack and damage DNA, and as a result, the combination of these two toxins would yield less direct DNA damage but an increase in intracellular ROS. To confirm this hypothesis, very thorough mechanistic assays are needed, probably starting with the characterization of the CYP or CYPs involved in AFB1 and OTA metabolization in Hep G2 cell line, and finding its inhibitors.

Conclusion

In conclusion, AFB1 and OTA turned out to be more cytotoxic in combination than alone in Hep G2, forming additive combination. As expected, AFB1 was genotoxic but OTA gave negative results in Hep G2. Surprisingly, the co-exposure with OTA decreased AFB1 genotoxicity; it could be that there was some kind of competition for the CYPs involved in mycotoxin metabolization. *In vitro* assays, even when using human cell lines, are always hard to extrapolate to *in vivo* real situations; however, additive and/or antagonic toxic effects may also be presumed *in vivo*. Therefore, further investigations are needed in order to improve risk assessment.

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Chapter 4

A poliphenol-enriched cocoa extract reduces free radicals produced by mycotoxins

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Resumen

Los polifenoles se caracterizan por la presencia de unidades fenol en su molécula. Estos compuestos pueden tener propiedades antioxidantes frente a especies reactivas de oxígeno (ROS) del tipo radicales libres. Se ha obtenido a partir de semillas de cacao un extracto enriquecido en polifenoles (PECE) con un 28% de procianidinas, la mayoría oligómeros de epicatequina. El PECE fue muy activo frente a los radicales: ácido 2,2'-azinobis-(3-etilbenzotiazolin-6-sulfonico (ABTS), 1,1-difenil-2-picrilhidrazilo (DPPH) y tris-(2,4,6-tricloro-3,5-dinitrofenil)metilo (HNTTM); y el ensayo del tris-(2,3,5,6-tetracloro-4-nitrofenil)metilo (TNPTM) reveló que el PECE no se comportaría como prooxidante. Por todo ello se consideró que sería un buen candidato como antioxidante en modelos *in vitro*. El extracto fue levemente citotóxico en Hep G2 e indujo ROS de forma dosis dependiente sólo a altas concentraciones cerca del límite de solubilidad. Las propiedades antioxidantes se ensayaron en Hep G2 tratadas con aflatoxina B1 y/o ocratoxina A. El PECE no fue efectivo frente a la AFB1, pero aumentó la viabilidad celular y redujo significativamente la cantidad de ROS en células tratadas con OTA o mezclas AFB1+OTA. Estos resultados concuerdan con el mecanismo oxidativo de la OTA e indican que los polifenoles del cacao son buenos candidatos como agentes antioxidantes.

Abstract

Polyphenols are characterized by the presence of phenol units in the molecules. These compounds may show antioxidant ability by scavenging reactive oxygen species (ROS) of the free radical type. A polyphenol enriched cocoa extract (PECE) was obtained from cocoa seeds with 28% of procyanidins which were mainly epicatechin oligomers. PECE was very active as free radical scavenger against 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and tris-(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) radicals; and the tris-(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) assay showed that the PECE might not be pro-oxidant. Thus it was considered a good candidate to be tested in *in vitro* models. It showed mild cytotoxic power on Hep G2 cells and induced ROS in a dose-dependent manner being weak oxidant only at high concentrations near the limit of solubility. The antioxidant properties were assayed in Hep G2 treated with the mycotoxins aflatoxin B1 (AFB1) and/or ochratoxin A (OTA). The PECE was not effective against AFB1 but it increased the cell viability and reduced significantly the amounts of ROS in cells treated with OTA or mixtures of AFB1+OTA. These results are coherent with the role of oxidative pathways in the mechanism of OTA and indicate that polyphenols extracted from cocoa may be good candidates as antioxidant agents.

Introduction

Polyphenols are secondary metabolism products of plants and constitute one of the most numerous and widely distributed groups of natural antioxidants in the plant kingdom. They can be divided into at least 10 different classes depending on their basic structure, being the most important ones that of flavonoids (Wollgast and Anklam, 2000). Flavonoids act as antioxidants via several mechanisms including the scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes (Cos *et al.*, 2004).

Red wine, cranberries, apples, tea and cocoa are among the richest food sources of flavonoids. Specifically, cocoa is rich in flavan-3-ol monomers, (–)-epicatechin (EC), (+)-catechin (C) and their related oligomers (procyanidins) (Osman *et al.*, 2004). The mean estimated daily intake of polyphenols in the diet is 1 g/person/day (Scalbert *et al.*, 2002). It has been estimated that about 90% of dietary polyphenols are bioaccesible in the small and in the large intestine (Saura-Calixto *et al.*, 2007). Monomeric (epi)catechins are rapidly metabolized to glucuronide and methyl conjugates in the small intestine, absorbed into the bloodstream and then further conjugated (glucuronidated, sulfated) in the liver. Procyanidins that are not absorbed in the small intestine are partially depolymerized into monomeric units and the remaining polymers reach the colon where they are metabolized by colonic microbiota into smaller compounds such as phenolic acids, which pass into the bloodstream and are finally excreted in urine. An array of phenolic metabolites are absorbed in the intestine and may contribute significantly to antioxidant and other physiological effects in the gastrointestinal tract and other tissues (Spencer, 2000; Laurent and Rios, 2003; Kwik-Uribe and Bektash, 2008; Urpi-Sarda *et al.*, 2009; Touriño *et al.*, 2011).

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are secondary products of several fungi metabolism mainly found in cereals, grapes, coffee, species, and cocoa and they belong to the most frequently occurring mycotoxins (Sedmíková *et al.*, 2001). The IARC classified AFB1 and OTA as class 1 (human carcinogen) and class 2B (possible human carcinogens), respectively (IARC, 1987; IARC, 1993; IARC, 2002). AFB1 is bioactivated in the liver by P450 cytochrome and the resulting epoxide metabolite causes the DNA damage by adduct formation (McLean and Dutton, 1995). A secondary route for AFB1 metabolism involves ROS formation but the mechanisms are still unknown (Bedard and Massey, 2006). Due to the carcinogenic nature of AFB1, the authorities have not yet estimated a safe intake and advise the ALARA principle (as low as reasonable achievable).

OTA is a potent nephrocarcinogenic compound in rodents but despite the controversy, it is considered an indirect genotoxic agent (Arbillaga *et al.*, 2004; Arbillaga *et al.*, 2007; Mally *et al.*, 2004; Mally *et al.*, 2005; Mally and Dekant, 2005; Turesky, 2005). OTA mechanisms of action have not been clearly determined but apparently its ability to generate reactive oxygen species (ROS) may explain the lipid, protein and DNA damage (Ringot *et al.*, 2006). Different official organizations have suggested safety PTDI between 5 and 17 ng of OTA per kg of body weight. With regard to AFB1 and OTA synergic behavior, it was found that OTA increased the mutagenicity of AFB1, and the mixture of both not only showed cytotoxic additive effects but also a slight increase in DNA fragmentation when compared to mycotoxins taken separately in Vero cells (El Golli-Bennour *et al.*, 2010). In Hep G2 cells, the mixture induced more ROS than each mycotoxin alone but OTA reduced the AFB1 genotoxicity (Corcuera *et al.*, 2011).

Recently, attention has been focused on inhibitory roles of natural constituents in suppressing or reducing the toxic action of mycotoxins, and results are not conclusive. In the case of AFB1, Muto *et al.* (2001) found that epicatechin (EC) partially inhibited the bioactivation of AFB1 in *Salmonella enterica serovar Typhimurium* which expressed human CYPs. However, Lee *et al.* (2001) could not find any inhibition in the epoxide synthesis when AFB1 was mixed with mouse liver microsomal proteins and catechins. Yet a cocoa polyphenol extract was able to suppress acrylamide toxicity, which has the same bioactivation pathway as AFB1, by improving the redox status and by blocking the apoptotic pathway activated by acrylamide (Rodriguez-Ramiro *et al.*, 2011). With regard to OTA, Costa *et al.* (2007) reported cytoprotective effects of catechins *in vitro* from OTA-induced cell damage and a good scavenging power according to inhibition of ROS production. Moreover, Baldi *et al.* (2004) found a small but significant protection (10 %) of α-tocopherol towards OTA-induced cell death in bovine mammary epithelial cells. However, Hundhausen *et al.* (2005) tested the effect of different polyphenols on OTA-induced cytotoxicity in Hep G2 liver cells, concluding that these compounds did not counteract OTA-induced cytotoxicity in working conditions.

In this work, a polyphenol-enriched cocoa extract (PECE) has been prepared and evaluated as an antioxidant agent in a cell free system and in Hep G2 cells. Once the antioxidant activity was confirmed, its ability to reduce AFB1 and OTA cytotoxicity and ROS induction has been tested showing promising results.

Materials and methods

Chemicals

Cocoa beans were provided by a national cocoa import factory. Acetone, acid acetic and chloridric acid were purchased from Panreac (Barcelona, Spain). 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%), 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) crystallized diammonium salt, (+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin-3-O-gallate (ECG), OTA, AFB1 standards and cysteamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-β-(2-aminoethylthio)catechin (Cya-Cat), 4-β-(2 aminoethylthio)epicatechin (Cya-EC), 4-β-(2-aminoethylthio)epicatechin-3-O-gallate (Cya-ECG) were synthesized and purified from grape extracts as previously described (Torres and Bobet, 2001). Tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) and tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) radicals were synthesized as described (Torres *et al.*, 2003; Torres *et al.*, 2007). For HPLC analysis, HPLC grade acetonitrile (Scharlab, Barcelona, Spain) and trifluoroacetic acid (TFA) (Fluorochem, Drebyshire, UK) were used. HPLC grade methanol was purchased from Merck (Darmstadt, Germany).

PECE preparation

OTA-free cocoa beans (20 g) were milled and lipidic compounds were removed with hexane. Next, theobromine and related compounds were discarded with ethanol:water:trifluoroacetic acid (15:84.9:0.1 v/v/v) (3 × 400 mL). Then, polyphenols were extracted with a mixture of acetone:water:acetic acid (70:29.98:0.02 v/v/v) (3 × 400 mL), the solid discarded by centrifugation and decantation and the acetone in the solution evaporated under vacuum. Finally, the polyphenolic extract was freeze-dried and kept in the dark in a N_2 atmosphere to avoid product oxidation.

Estimation of polyphenolic composition

The size and composition of the procyanidins were determined by HPLC analysis (Torres and Selga, 2003). Depolymerized sample was analyzed by HPLC-DAD using a Hitachi Lachrom Elite HPLC (San Jose, CA, USA) fitted to a Kromasil C18 column (25 × 0.4 cm, 5 μm) from Teknokroma (Barcelona, Spain). Components were eluted with a binary system 0.1% (v/v) TFA in HPLC grade

water [A] and 0.1% TFA (v/v) in acetonitrile [B], gradient 9.6 to 20% [B] in 22 min, flow rate 1 mL/min. Selected wavelength for the polyphenol quantitative analysis was 214 nm.

Using this methodology, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of Cya (cysteamine) whereas the extension moieties were released as the Cya derivatives on the fourth position of the flavanoid system. Calibration curves of terminal units C and EC and extension units Cya-Cat and Cya-EC were plotted by analyzing 6 standards within the working range in triplicate. Then the terminal and extension units content in the cocoa extract were determined and the mean degree of polymerization (mDP) and the mean molecular weight of the polymer (mMW) were calculated as follows:

$$mDP = \frac{Total\ nmol(Cat + Ec + Cya - Cat + Cya - EC)}{Total\ nmol\ ter\ \min al\ units(Cat + Ec)}$$

$$mMW = \frac{Total\ ng(Cat + Ec + Cya - Cat + Cya - EC)}{Total\ nmol\ ter\ \min al\ units(Cat + Ec)}$$

Free radical scavenging activity

To study the free radical scavenging activity, the ABTS, DPPH, HNTTM, TNPTM methods described in Touriño *et al.* (2008) were applied.

ABTS assay measures the total antioxidant activity, comparing antioxidant activity of the extract with that of Trolox. In this case, the concentrations of cocoa polyphenolic extract (50 μL) used to mix with the 1 mM ABTS solution (1950 μL) were 50, 62.5, 75, 100 and 125 μg/mL in methanol. The Trolox equivalent antioxidant activity (TEAC) of the fractions was expressed as μmol of Trolox equivalent to 1 mg of cocoa polyphenolic extract.

The free radical scavenging power was also evaluated against two other radicals: DPPH and HNTTM. These two radicals are used to calculate the stoichiometry of the redox reaction and to discriminate between hydrogen donation and electron-transfer mechanisms of radical scavenging. While the DPPH assay measures the combined hydrogen donation and electron-transfer capacity of the polyphenols, the HNTTM stable radical exerts its action exclusively by electron transfer. For both DPPH and HNTTM assays, the antiradical power (ARP) is expressed as the inverse of ED_{50} (μg of extract able to consume one-half the amount of free radical divided by μmol of initial DPPH or HNTTM). The concentrations of cocoa polyphenolic extract to be mixed with the radical solution

were 20, 70, 100, 150, 200 and 300 μg/mL of methanol in the case of 60 μM DPPH and 0.5, 2.5, 5, 15, 35 and 50 μg/mL of chloroform:methanol 2:1 in the case of 120 μM HNTTM.

The specific reactivity of TNPTM radical with the most reducing positions (e.g. pyrogallol group) was compared with the hydrogen donation ability (DPPH) and global electron transfer capacity (HNTTM) in order to provide valuable information regarding the ability of some components to engage in putatively prooxidant/toxic effects involving electron transfer to oxygen. The concentrations of PECE to mix with the 80 μM TNPTM solution were 32.5, 65, 97.5 and 130 μg/mL of chloroform:methanol 2:1.

Cell cultures

Hep G2 (hepatocellular carcinoma epithelial cell line) (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). It was kept in the laboratory satisfying the requirements for Good Laboratory Practice (GLP) conditions. A master bank was created on arrival and a working bank was created to perform the assays of this study, controlling the passing number, which never surpassed 20.

Cells were cultured in monolayer in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Prat de Llobregat, Barcelona, Spain) supplemented with 10% of fetal calf serum (FCS, Gibco) and 1% of antibiotics (10,000 U/mL penicillin and 10,000 μg/mL streptomycin, Gibco). Cells were grown at 37ºC and 5% CO_2 in a humidified atmosphere.

Cytotoxicity assay

Cell viability was assessed by the MTT assay that measures the reduction of MTT (3-(4,5-dmethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) to formazan by the mitochondrial enzyme succinate dehydrogenase (Mosmann, 1983). Cells were seeded on 96-well plates and after 24 h of incubation, cells were washed with PBS and exposed for 24 h to: PECE (5, 25, 50, 75 and 100 mg/L), AFB1 (1.2, 6 and 30 μM), OTA (50, 100 and 200 μM), AFB1 (1.2, 6 and 30 μM)+50μM OTA. To study the protection of PECE against mycotoxins, cells were pretreated during 24 h with PECE (25 or 50 mg/L), and then, the same concentrations of AFB1, OTA or AFB1+ 50μM OTA were used. Plates were washed and MTT (5 mg/mL) was added to fresh culture medium to each well and incubated another 2 h. The supernatant was removed, and the insoluble formazan crystals were dissolved with 100 μL of DMSO. The absorbance was measured at 540 nm using a

spectrophotometer reader (Titertek Multiskan® MCC/340 MK II). Results were expressed as the percentage of viability (%) with respect to the control (solvent treated cells) according to the following formula:

$$Viability\ (\%) = \frac{Absorbance_{treated\,cells} - Absorbance_{blank}}{Absorbance_{control\,cells} - Absorbance_{blank}} \times 100$$

Cells survival values above 70-80% were considered non cytotoxic.

Dichlorofluorescein assay

ROS were determined by using fluorescent probe DCFH-DA (2',7'-dichlorfluorescein-diacetate), as Wang and Joseph described (Wang and Joseph, 1999). DCFH-DA crosses the membranes and is enzymatically hydrolyzed by intracellular esterases in order to form non fluorescent DCFH (dichlorofluorescein). The latter is rapidly oxidized to form highly fluorescent DCF (dichlorofluorescein) in the presence of ROS. The DCF fluorescence intensity parallels the amount of intracellular ROS. Hep G2 cells were incubated for 30 min at 37ºC in serum-free and phenol red lacking DMEM medium containing 100 mM DCFH-DA. Afterwards, cells were washed once with PBS and exposed during 24 h to: PECE, AFB1, OTA, AFB1+50μM OTA or to a pretreatment of PECE during 24 h and then AFB1, OTA or AFB1+50μM OTA, using the same concentrations as in previous section. ROS were measured using a microplate fluorometer Fluoroskan Ascent (Thermo Labsystems) at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. Afterwards, a MTT assay was performed in order to obtain the exact % of cell survivor in each well. The value of fluorescence was divided per the % of cell survivor at each concentration in order to obtain the relative fluorescence (RF), which is independent of the cell viability. Therefore, the relative fluorescence of the negative control would be 1, and values over 1 suggest an increase of intracellular ROS.

Solubility and interactions

The polyphenol extract was dissolved in $NaHCO_3$ (Riedel-deHaën, Seelze, Germany) at pH 7.4 using an ultrasound bath without exceeding 30ºC. The maximum concentration reached was 100 mg/L. OTA was dissolved in 0.1 M $NaHCO_3$ (pH 7.4) (Riedel-deHaën, Seelze, Germany) to an initial concentration of 2.48 mM and AFB1 was dissolved in DMSO (Panreac Quimica SAU, Barcelona, Spain) to an initial concentration of 15.15 mM. All solutions were aliquoted and

maintained at -20ºC until use. PECE and AFB1 solutions were kept in the dark to avoid light degradation. The experiments were performed without fetal calf serum (FCS) in order to avoid serum interactions with the PECE or mycotoxins binding to proteins.

Statistical analysis

Three independent experiments were performed to check cytotoxicity and ROS induction. Data is presented by descriptive analysis [mean±standard deviation (SD) of the replicated experiments]. Comparisons were performed by non-parametric Kruskal-Wallis H-test and Mann-Whitney U-test. $P \leq 0.05$ was accepted as the level of significance.

Results

PECE preparation and mean polyphenol composition

A water:acetone 3:7 cocoa extract containing C and EC monomers and oligomers was prepared as described previously. Calibration curves of C, EC, Cya-Cat and Cya-EC were prepared covering the working range. Six standards of each compound were analyzed and a linear response versus concentration was obtained. Sample analyses were carried out in triplicate having a RSD < 8%. Cocoa extract had 28 % of procyanidins being richer in EC (89%) than in C (11%). The amount of terminal EC (12%) is clearly lower than the amount of chain EC (77%) with the mean oligomeric size (mDP) being 4.5 units and the mean molecular weight (mMW) 1,300 g mol^{-1} (see table 1).

Free radical scavenging activity in cell-free systems

The free radical scavenging power was evaluated against stable radicals that measure the electron and proton transfer capacity (ABTS assay and DPPH assay) and radicals which are exclusively quenched by electron transfer to an anion with subsequent slow proton incorporation (HNTTM assay). Additionally, pro-oxidant activity of extracts was tested using the TNPTM which measures the electronic transfer capacity of ROS inducing compounds. Results are presented in table 2.

Cytotoxicity and ROS production

Five different concentrations up to the maximum soluble one were used to test the cytotoxic effects of PECE. A dose-dependent curve was observed after 24 h of treatment and the cell survival was over 50% for the entire concentration range (figure 1A); therefore, the IC50 could not be calculated.

In order to investigate the possible induction of intracellular ROS, the same five PECE concentrations were chosen. Significant increases of ROS were observed in a dose-dependent relationship (figure 1B).

PECE protection against mycotoxin toxicity

With the aim of testing the PECE protection capability against AFB1 and OTA toxicity, Hep G2 cells were incubated with the extract during 24 h prior to mycotoxin treatments. Two PECE concentrations were chosen: 25 and 50 mg/L. Cell viability of Hep G2 decreased with respect to the control after being treated with AFB1 or OTA or a mixture of AFB1 (same concentrations as in single treatments) + 50 μM OTA. PECE pretreatment increased AFB1 induced cytotoxicity (figure 2A), whereas OTA and AFB1 +OTA cytotoxicity were partially counteracted by the pretreatment of PECE (figure 2 C, D, E and F).

Table 1: Mean oligomeric size of procyanidin units (mDP) and percentages of the different flavonoids in extracts from cocoa and different sources.

Source of the polyphenolic crude extract	mDP	% procyanidin	Catechin		Epicatechin		References
			% terminal	% chain	% terminal	% chain	
Theobroma Cocoa seeds (PECE)	4.5	28	10	1	12	77	Present work
Pinus Pinaster	2.3	17	40	13	5	42	Touriño et al., 2005
Pinus Radiata	2.9	36	34	49	5	12	Jerez et al., 2007
Hammamelis Virginiana	2.9	5.8	3.4	6.7	7.9	6.1	Touriño et al., 2008

Table 2: Results of the free radical scavenging activity assays.

Assay	Parameter	Value
ABTS	TEAC	0.28
DPPH	ED_{50}	91
	ARP	11
HNTTM	ED_{50}	91
	ARP	11
TNPTM	ED_{50}	---

TEAC (trolox equivalent antioxidant capacity): mg of extract equivalent to 1 mM of Trolox.

ED_{50}: µg of PECE able to consume 1/2 of radical divided by µmol of initial radical.

ARP (antiradical power): inverse of $ED_{50} \times 1000$.

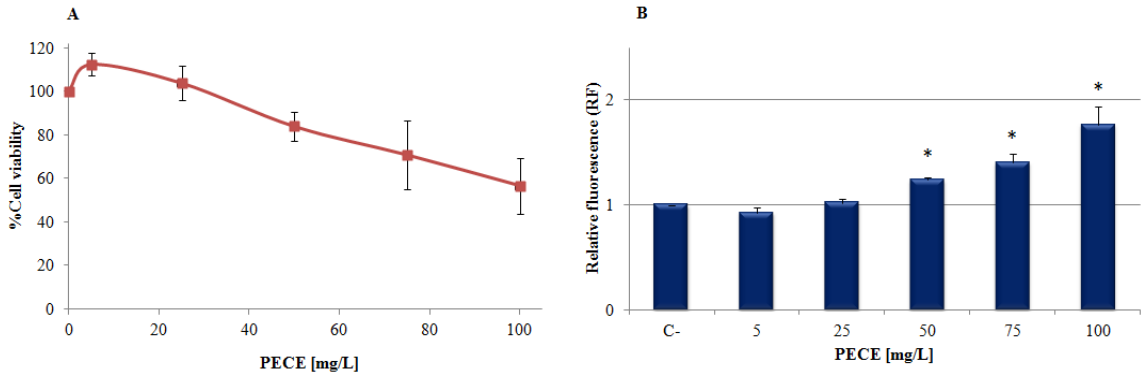

Figure 1: A) viability curve of Hep G2 after being treated during 24 h with PECE obtained with the MTT assay. B) Intracellular ROS induction in Hep G2 after 24 h with PECE. RF values were compared with control levels (C-) (p≤0.05). The mean and SD of 3 independent experiments are shown.*

The ROS scavenging effects against AFB1 and OTA alone and combined had also been tested with a pre-treatment of 24 h with two different concentrations of PECE: 25 or 50 mg/L. The extract showed protection against AFB1 ROS induction at the concentration of 50 mg/L, restoring the ROS levels reached at 30 µM of AFB1 to those of the control cells (figure 3B). When the cells were incubated with OTA, a reduction in the ROS was observed with 25 mg/L (figure 3C) and a weak protection was observed with 50 mg/L (figure 3D). In the combined treatments, the ROS reduction was also more pronounced with the low PECE concentration (figure 3E); in addition, significant reductions were observed with 50 mg/L of PECE (figure 3F).

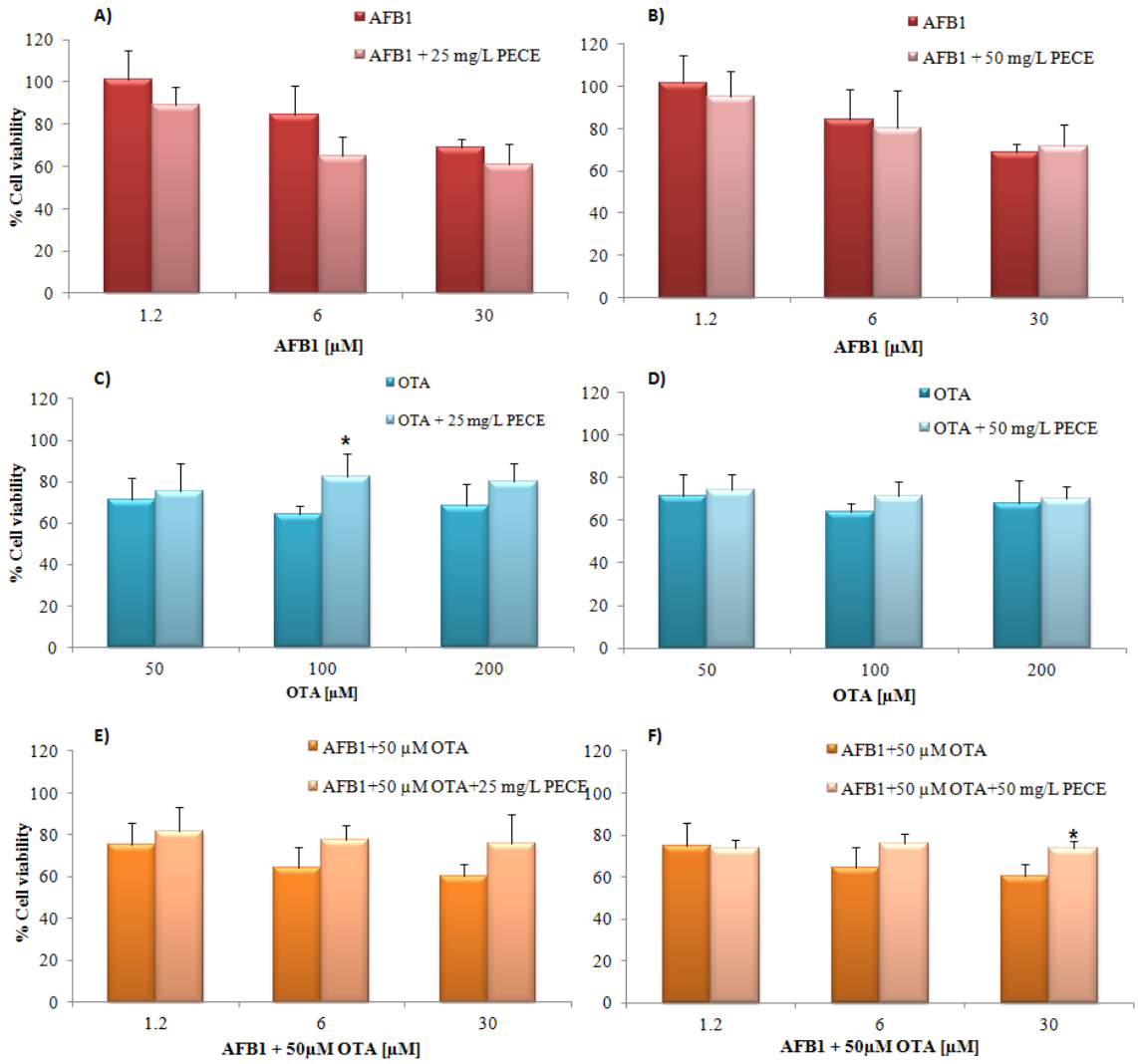

Figure 2: Comparison between cell viability of Hep G2 after 24 h of treatment with AFB1, OTA or the mixture of AFB1+50 µM OTA and the same treatments with a previous protection of PECE (25 or 50 mg/L) during 24 h. In order to observe the significant effects of PECE, the results between pretreated with PECE and non-pretreated have been compared statistically (p≤ 0.05). The mean and SD of 3 independent experiments are shown.*

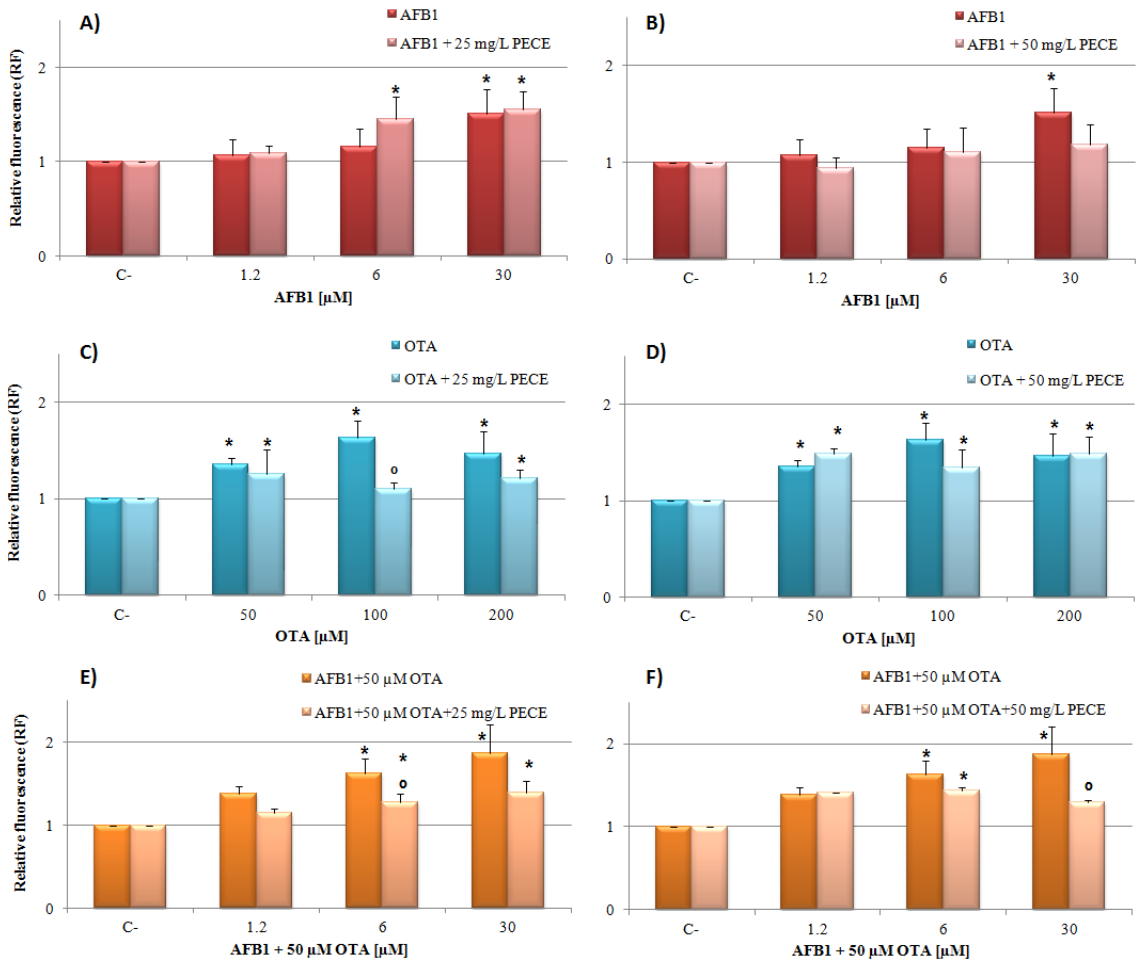

Figure 3: ROS induction in Hep G2 of AFB1, OTA and a mixture of AFB1+50 µM OTA and the protective effects of a pretreatment of PECE (25 or 50 mg/L) during 24 h against mycotoxins. The ROS induction of each treatment has been compared to the basal level (C-) ($p \leq 0.05$). The significant ROS reduction due to the pretreatment with PECE has been indicated with the ° symbol when $p \leq 0.05$. The mean and SD of 3 independent experiments are shown.*

Discussion

Baba *et al.* evaluated the levels of EC and its metabolites in plasma and urine after intake of chocolate or cocoa by male volunteers. They found that EC was partly absorbed and present in plasma in the free form or as a component of various non-methylated and methylated conjugates (Baba *et al.*, 2000). Cocoa polyphenols and alkaloids comprise approximately 14 – 20% of the whole

cocoa bean weight depending on the processing conditions (Nazaruddin *et al.*, 2006). EC monomers and oligomers constitute as much as 60% of the total polyphenol content in cocoa (Dreosti, 2000).

In the extract prepared for this work the proportion of procyanidins was 28%, where EC monomers and oligomers constituted 89%. Comparing with other phenolic extracts from different plant sources (*Pinus Pinaster* (Touriño *et al.*, 2005), *Pinus Radiata* (Jerez *et al.*, 2007) or *Hammamelis Virginiana* (Touriño *et al.*, 2008)), the PECE had the largest degree of polymerization and EC content (see table 1). According to the free radical scavenging activity, the PECE was more efficient than Trolox and as efficient as other polyphenol enriched extracts prepared by the authors such as pine bark (Touriño *et al.*, 2005). The ED_{50} value obtained in the DPPH and the HNTTM is directly proportional to the hydrogen and the electron-transfer ability, respectively. In the case of the PECE both ED_{50} values are the same. As the electron transfer capacity may imply the presence of pro-oxidant compounds inducing the formation of ROS, the TNPTM assay was carried out. Electron-transfer via pro-oxidant compounds such as pyrogallols were not detected during this test. Thus, the PECE, with a high content in EC and 4.6 of mDP, was selected as a promising antioxidant candidate for the reduction of the damage produced by AFB1 and OTA.

These antioxidant properties encouraged us to test the extract in a biological system, and the Hep G2 cell line was selected. Hep G2 cell line has been proposed as a good experimental model for detecting mutagenicity and antimutagenicity (Knasmuller *et al.*, 1998) and for studying metabolism-induced drug hepatotoxicity (Otto *et al.*, 2008) because it retains some phase I and phase II metabolizing enzymes. Cytotoxicity through the MTT assay was evaluated. The PECE was a weak cytotoxic mixture because at the limit of solubility, cell survival was higher than 55%. This may be due to the absence of gallate groups which have been related with apoptotic induction (Lizarraga *et al.*, 2007). Also, pine extracts which have procyanidin oligomers and are not galloylated showed a low cytotoxic potency (Ugartondo *et al.*, 2007).

It has been reported that some chemopreventive agents with antioxidant properties (alpha-tocopherol, quercetin, catechins, isothiocyanates, N-acetylcysteine) could act as prooxidants, causing DNA damage via generation of reactive oxygen species in the presence of metal ions and endogenous reductants (Kawanishi *et al.*, 2005). Azam *et al.* found out that EC and EGCG had prooxidant properties such us as the generation of superoxide anion and the hydroxyl radical. Both polyphenols lead to oxidative DNA cleavage in the presence of copper ions (present in chromatin) (Azam *et al.*, 2004). Even though the TNPTM assay showed that the PECE might not be pro-

oxidant, the possible induction of ROS was studied in Hep G2, and a dose-response ROS increase was detected only at high concentrations. For this reason, the two concentrations that were selected for studying PECE protection against mycotoxin toxicity were: a noncytotoxic concentration which did not induce ROS (25 mg/L) and the tested concentration that induced a slight cytotoxic effect (around 80% of cell survival) (50 mg/L). This concentration induced very low but significant levels of ROS; therefore, this concentration might not be efficient enough in cell protection. However, 50 mg/L was selected due to the fact that antioxidant/prooxidant balance of polyphenols at cellular levels may be modulated by the presence of other substances (Azam *et al.*, 2004; Chendea *et al.*, 2010).

The protective treatments with PECE can be performed before, during or after treatment with mycotoxins. Costa *et al.* (2007) found excellent protective effects of pure catechin derivatives (EGCG and ECG) against OTA cytotoxicity, acting as good ROS scavengers. This effect was detected when the antioxidants were added to LLC-PK1 cells (pig kidney cell line) 24 h before the mycotoxin with no effects in the co- and post-treatments (Costa *et al.*, 2007). Therefore, Hep G2 cells were incubated with PECE during 24 h before they were treated with the mycotoxins. Regarding AFB1, the PECE did not reduce the cytotoxicity. Lee *et al.* (2001) observed that catechins have no inhibitory effects on the biotransformation of AFB1 to its epoxide. Moreover, the PECE was not very effective on ROS scavenging and only reduced the ROS levels at the highest concentration with 50 mg/L. This may indicate that radical formation is not the principal mechanism of AFB1 cytotoxicity, although it does play a role as a secondary route of action (Bedard and Massey, 2006). Regarding OTA cytotoxicity, both in isolated and combined treatments with AFB1, the cell survival was improved, in particular with 25 mg/L of PECE, even when AFB1 was present. The protection was not efficient with 50 mg/L in OTA treated cells, but at least the cell death did not increase in the presence of PECE. In concordance with this, 25 mg/L of PECE was very effective reducing ROS formation in all the OTA and combined treatments. Surprisingly, the greater reduction of ROS was observed with the "prooxidant" concentration of 50 mg/L in the combined treatment of 30 μM AFB1+50 μM. This observation highlights the dual pro/anti oxidant activity of the molecules with multiple phenol units according to the redox properties of the molecules that interact with them. In conclusion, a PECE was prepared and tested as antioxidant agent. It had encouraging antioxidant properties in the cell free assays and low cytotoxic power in Hep G2 cells. The PECE reduced ROS generated by the mycotoxins, particularly in isolated OTA treatments or combined AFB1 and OTA treatments. Thus, polyphenols extracted from cocoa have a good

antioxidant activity and may be efficient reducing the generation of ROS produced by mycotoxins or other oxidant agents.

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Chapter 5

Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin A in rat plasma, liver and kidney

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Resumen

Se ha validado un método UHPLC-FLD rápido y sencillo para la cuantificación simultánea de AFB1 y OTA en plasma, hígado y riñon de rata de acuerdo con los siguientes criterios: selectividad, estabilidad, linealidad, precisión, exactitud, recuperación, robustez y límites de quantificación y detección. El método de extracción, las curvas de calibrado y las condiciones cromatográficas fueron comunes para las tres matrices. Las micotoxinas se extrajeron del plasma y de los homogeneizados de tejido (100 μL) con una mezcla de acetonitrilo:ácido fórmico (99:1) (300 μL). La separación cromatográfica se realizó con un gradiente de agua y acetonitrilo:metanol (50:50), ambas acidificadas con 0,5% de ácido fórmico. El método no utiliza columnas de inmunoafinidad y permite reducir los volúmenes de muestra requeridos así como los residuos tóxicos. La detección esta basada en una reacción fotoquímica postcolumna que aumenta la respuesta de la AFB1 sin afectar a la OTA. La recuperación de las micotoxinas para cada matriz fue muy eficiente, entre 93- 96% para la AFB1 y entre 94 - 96% para la OTA. Para ambas micotoxinas los LCs fueron 2 μg/L en plasma y 8 μg/kg en hígado y riñón. El método fue satisfactoriamente aplicado en muestras de rata administradas con una dosis oral de una mezcla de AFB1 y OTA, y puede ser una herramienta útil en estudios toxicocinéticos y toxicológicos.

Abstract

A rapid and simple method for the simultaneous quantification of AFB1 and OTA in rat plasma, liver and kidney by UHPLC-FLD has been successfully validated according to the following criteria: selectivity, stability, linearity, precision, accuracy, recovery, robustness and limits of quantification and detection. The extraction method, calibration curves and chromatographic conditions are common for the three matrices. Plasma and homogenized tissue samples (100 μL) were extracted with acetonitrile-formic acid mixture (99:1) (300 μL). Chromatographic separation was performed with a mixture of water and acetonitrile:methanol (50:50), both acidified with 0.5% of formic acid using a gradient profile. The method avoids the use of immunoaffinity columns and allows reduction of sample and solvent volumes as well as toxic wastes. The detection is based on a photochemical reaction which enhances the AFB1 response without affecting the OTA signal before reaching the fluorescent detector. The mycotoxin recovery for each matrix was very efficient, between 93 - 96% for AFB1 and between 94- 96% for OTA. For both mycotoxins the LOQs were 2 μg/L in plasma and 8 μg/kg in liver and kidney. The method has successfully been applied to rat samples after a single oral administration of a mixture of AFB1 and OTA and it could be a useful tool in toxicokinetic and toxicological studies.

Introduction

Mycotoxins are small (MW~700 amu) secondary metabolites produced by different fungal species that can contaminate many agricultural commodities during harvest and/or while in storage. Most of mycotoxins are produced by *Fusarium* (pre-harvest), *Penicillium* (post-harvest) and/or *Aspergillus* (pre/post harvest) fungi. They can reach human beings through contaminated food, as well as via edible products (milk, eggs, meat, blood, etc) obtained from animals fed with contaminated feeds (Soriano del Castillo, 2007). Due to the globalization of the trade in agricultural commodities, mycotoxins may currently appear in any developed or developing country in local or imported products.

Aflatoxins and ochratoxin A (OTA) belong to the most frequently occurring mycotoxins (Sedmíková *et al.*, 2001). The IARC classified Aflatoxin B1 (AFB1) and OTA as class 1 (human carcinogen) and class 2B (possible human carcinogen), respectively (IARC, 1987; IARC, 1993; IARC, 2002). Aflatoxin B1 is genotoxic *in vivo* and *in vitro*; its target organ is liver but it may cause tumors in other organs such as colon and kidney (McLean and Dutton, 1995; Newberne and Butler, 1969). Ochratoxin A is a potent nephrocarcinogenic compound in rodents but despite the controversy with regard to its mechanisms of action, some existing research suggests that it is an indirect genotoxic agent. Moreover, OTA is nephrotoxic, hepatotoxic, teratogenic and immunotoxic, and recent studies have related it to neurodegenerative diseases such as Parkinson and Alzheimer (Ringot *et al.*, 2006; Pfohl-Leszkowicz and Manderville, 2007). Due to the aforementioned, a Tolerable Weekly Intake (TWI) of 120 ng/kg of body weight has been established for OTA (EFSA, 2006) but, as of yet there is no threshold for AFB1; therefore, ALARA (as low as reasonably achievable) principle limit must be applied as it is not possible to identify an intake without risk (EFSA, 2007).

Human population is chronically exposed to multiple mycotoxins because of several reasons. First, the same food might be contaminated by more than one mycotoxin as the co-occurrence of AFB1 and OTA in edible products has been demonstrated; some examples are in dried fruits and figs (Bircan, 2009), in paprika (Shundo *et al.*, 2009; Santos *et al.*, 2010), and in breakfast cereals (Villa and Markaki, 2009). Moreover, since the human diet is varied, mycotoxins might reach humans from different pathways; and, finally, mycotoxins are thermostable and can remain in food even after the fungus has been removed (Soriano del Castillo, 2007). Co-exposure to different mycotoxins, could originate synergic or additive toxic effects on human or animal health; however, knowledge regarding

this aspect or regarding the influence of co-ocurrence on toxicokinetic or toxicological characteristics of the mycotoxins is still scarce.

Reference literature reports numerous methods for individual quantification of AFB1 or OTA, most of which are applied to foodstuffs, but also applied to animal matrices such as poultry (Gregory III *et al.*, 1983), fish meat (Papp *et al.*, 2002), eggs (Herzallah, 2009), milk (Herzallah, 2009; Valenta, 1998), swine (De Saeger *et al.*, 2004), and meat and meat products (Herzallah, 2009; Valenta, 1998). Nevertheless, very few of these studies are suitable for application in complex biological samples and/or during toxicological studies because they use large volumes of sample, which is not easily available when working with laboratory animals. Moreover, most of these studies do not report validation data, which is very important so as to provide evidence of the reliability of the results (Bressolle *et al.*, 1996).

The development and validation of methods for the simultaneous determination of the most important mycotoxins in biological matrices would be very useful because they would be adequate tools for toxicokinetic and toxicology studies that investigate the effects of co-exposure, minimizing the cost of the analysis and the use of laboratory animals.

In this paper, a UHPLC-FLD method with simultaneous extraction and analytical quantification procedures for AFB1 and OTA and in rat plasma, kidney and liver has been validated and successfully applied in biological samples, demonstrating its usefulness for toxicological or toxicokinetic studies.

Materials and methods

Safety precautions

Aflatoxin B1 and ochratoxin A are toxic substances. They were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all procedures carried out and during the manipulation of treated animals or contaminated samples FPP3 masks were used.

Reagents

For the analytical standards, AFB1 was purchased as a solution of 2 mg/L in acetonitrile (ACN) and OTA was purchased as a solution of 10 mg/L in ACN, both from OEKANAL® Fluka

(Schnelldorf, Germany) as certified reference materials. For oral administration, mycotoxins were purchased in powder from Sigma (Steinheim, Germany) and they were dissolved in 0.1 M $NaHCO_3$ (Riedel-de Haën, Seelze, Germany), adjusted to pH 7.4 with HCl and maintained at -20ºC until their use. For the tissue homogenates, sodium phosphate buffer (0.05 M, pH 6.50) was prepared by adding 6.90 g of $NaH_2PO_4.H_2O$ (Merck, Darmstadt, Germany) to 900 mL of type II water. The pH of the dissolution was adjusted to 6.5 with NaOH (Agilent technologies, Waldbronn, Germany) and the volume was adjusted to 1 L. All reagents used for the HPLC analysis were of analytical grade. ACN and methanol (MeOH) HPLC grade and formic acid were obtained from Sigma Aldrich (St. Quentin Fallavier, France). Millipore type I water was obtained daily from a Milli-Q water-purifying system (Millipore, Bedford, MA, USA).

Standard solutions

Working standard solutions (mixture of AFB1 and OTA) were prepared by appropriate dilution of the commercial standards with ACN and MeOH to a proportion of 50:50 v/v. Four working solutions were prepared (750, 300, 30 and 3 μg/L of AFB1 and OTA) and stored at -20ºC. The calibration standards were prepared by evaporating volumes of working standard solutions (after being kept at room temperature during 30 min) under a stream of nitrogen and then the residues were dissolved in 200 μL of mobile phase. The calibration standards were kept in the injection tray at 4ºC without illumination until analysis.

Animals

The *in vivo* experiments were approved by the Ethics Committee on Animal Experimentation of the University of Navarra.

Ten-week-old male and female Fisher 344 (F344) rats, purchased from Harlan (Horst, The Netherlands), were used. On the day of arrival, the animals were weighed (weight variation did not exceed ± 20% (OECD, 1984; OECD, 2009)) and then distributed to polycarbonate cages with stainless steel covers for one week in order to allow acclimatization to the environmental conditions: 12 h day/night cycle, temperature 22 ± 2ºC, relative humidity 55 ± 10%, standard diet (Harlan Iberica, Spain) and water *ad libitum*.

Plasma, kidney and liver used as blank samples for the validation of the method were obtained from 12 non-treated animals (6 male and 6 female). In the application of the method, two male rats

were administered a single dose of a mixture of AFB1 and OTA (0.5 mg/kg of AFB1 and 0.1 mg/kg bw of OTA) by oral gavage. Samples of plasma, liver and kidney were extracted and analyzed.

Sample obtaining and mycotoxin extraction procedure

Sampling methods were based on those described by Vettorazi *et al.* (2008) for toxicokinetic studies of OTA in rat with some modifications. Blood from decapitation was collected in BD (Plymouth, UK) Vacutainer tubes (5.4 mg K3E, 3 mL) and centrifuged at 1266 x g for 15 min. The obtained plasma was aliquoted and stored at -80ºC until the extraction of mycotoxins was carried out. Liver and kidney were extracted from the animals, washed with water until the external blood was removed, and then blotted on filter paper. Pieces of each organ were cut and weighed. The medium weights of them (15-20% of relative standard deviation (RSD)) were: 1.64 g and 1.02 g of male and female livers respectively and 0.33 g and 0.23 g of male and female kidneys respectively. Afterwards, they were flash-frozen in liquid nitrogen and stored at -80ºC. In order to prevent cross contamination between samples, all the dissection material was cleaned with water and rinsed with ethanol after each animal necropsy.

For mycotoxin quantification, kidney and liver were homogenized for approximately 1 minute in a round-bottom plastic tube (sterile PP-tube, from Greiner bio-one GmbH (Frickenhausen, Germany)) with 4 μL of cold sodium phosphate buffer (0.05 M, pH 6.50) per mg of tissue in a Polytron PT 3,000 homogenizer with a metal rod (PT-DA 3012/2 TS Kinematic (Littau, Switzerland)). After each use, the homogenizer was cleaned with water, rinsed with ethanol and turned on until ethanol evaporation. The tissue homogenates were aliquoted (between 500 μL and 1mL) and stored for at least one day at -80ºC until the mycotoxin extraction was carried out.

Before the extraction step, plasma or tissue homogenates were kept at room temperature for 30 min and vortexed. Next, 100 μL of the sample (plasma or homogenates) was mixed with 300 μL of the extractive solution (ACN - 1% HCOOH), vortexed for 2 min and centrifuged at 6200 x g for 15 min at 4ºC in order to precipitate proteins. The supernatant (200 μL) was evaporated to dryness under vacuum (in a miVac DUO concentrator, Genevac (Ipswich, UK) during 15 min at 40ºC) and immediately afterwards the dry sample was resuspended in 200 μL of mobile phase and vortexed during 2 min. Plasma samples with expected high concentrations of OTA were diluted 1:20 in mobile phase. Dilution factor for plasma was 4 (80 in the case of high concentrated samples), and 16

for kidney and liver. The pH attained in the extraction step was approximately 2.6. Samples were filtered and placed in vials in the HPLC tray at 4ºC in darkness until injection

Apparatus and chromatographic conditions

UHPLC analyses were performed with an Agilent Technologies 1200 liquid chromatographic system equipped with a fluorescence detector (G1321A model) controlled by ChemStation B.03.02 software (Hewlett-Packard). Mycotoxins were separated on an Ascentis® Express C18 column (150 mm x 2.1 mm; 2.7 μm) from Supelco (PA, USA). The mobile phase was a mixture of an organic phase (A) (MeOH-ACN, 50:50, v/v) and water (B), both acidified with 0.5% of formic acid. The injection volume was 40 μL and the flow rate was 0.9 mL/min. Chromatography was performed at 60ºC. Proportion of both organic and aqueous phases was switching between isocratic and gradient profiles during the entire analysis procedure. The elution program starts isocratic until minute 2.4 with 30% of A, then from 2.4 to 2.5 min the organic phase increases up to 43%, from minute 2.5 to 8.3 min another isocratic profile at 43% of A, from minute 8.3 to 10.0 there is a last increase up to 65% of A and finally, from minute 10.0 the system returns to 30% of A to restore the starting conditions during 5 min. The retention times under these conditions were 2.5 min for AFB1 and 8.4 min for OTA. Before the sample entered the fluorescence detection cell, a photoderivatization device (AURA Industries, NY, USA) with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.25 mL (5 m x 0.25 mm) was included. During the first 4 min of analysis, fluorescence conditions were optimized for AFB1 (excitation 366 nm and emission 433 nm wavelengths), and after that for OTA (excitation 225 nm and emission 461 nm wavelengths)

The chromatographic separation was evaluated for each matrix at the limit of quantification level using the following parameters: retention time (t_R,) retention factor (k'), symmetry, peak width at half height (w_h), number of theoretical plates (N) and resolution (R_s).

Validation of the UHPLC-FLD method

The analytical method was validated for each mycotoxin according to the following method performance characteristics: selectivity, stability, linearity, precision and accuracy (within- and between-day variability), recovery (in intermediate precision conditions), limits of detection (LOD) and quantification (LOQ), and robustness. Selectivity, LOD, LOQ, recovery and stability were studied for each matrix. For this purpose, a pool of blank samples of plasma, kidney or liver obtained from 12 animals (6 male and 6 female) was used.

Selectivity of the method was improved by using a photochemical reactor (PHRED) before a fluorescence detector with blank plasma and tissue homogenates. However, the ability of the method to distinguish AFB1 and OTA from other endogenous components was evaluated for the 3 matrices by analyzing and comparing blank samples before and after being spiked with the mycotoxins at LOQ levels. Moreover, due to the fact that OTA basal levels were detected in plasma, samples that are naturally contaminated with OTA were analyzed and afterwards spiked with a standard solution in order to see an increase of the area of the corresponding peak. In addition, in both cases (naturally contaminated and spiked) the samples were reanalyzed after changing the % of organic component in the mobile phase in order to delay OTA peak and therefore be able to observe the presence or absence of broadening, shoulders or others interfering peaks.

The stability of mycotoxins was determined in the working solution, during storage of samples at -80ºC and in the chromatograph tray before analysis at 4ºC. Three concentrations of working solutions (1, 10 and 100 μg/L) stored at -20ºC were analyzed during 8 weeks. Mycotoxin stability in the HPLC chromatographic tray was evaluated for the three matrices by analyzing the extracts of spiked samples (40 μg/L plasma and 160 μg/kg liver/kidney) just after preparation and over a period of 15 h. Moreover, the stability of mycotoxins in plasma and in tissue homogenates stored at -80ºC has also been determined by analyzing spiked samples (40 μg/L plasma and 160 μg/kg liver/kidney) 24 h, 1 week, 1 month and 6 months after preparation.

For each mycotoxin, two calibration curves were plotted from 0.5 up to 150 μg/L using calibration standards. The range was split into two: a low calibration curve from 0.5 to 30 μg/L, with eight data points; and a high calibration curve from 30 to 150 μg/L with six data points. Three replicates of each calibration standard were analyzed. Within- and between-day precision and accuracy of the linearity were studied by analyzing three replicate calibrations standards at 0.5, 2, 15, 30, 90 and 150 μg/L on one day (within-day) and on three different days (between-day).

Due to the fact that adequate reference biological spiked materials were not available, fortification was carried out with known concentrations of mycotoxins in order to establish the recovery and precision of the method. AFB1 and OTA were added to blank plasma, liver or kidney homogenate pools in order to obtain 2, 8, 120, 600 μg/L in plasma, and 8, 32, 480, 2,400 μg/kg in organs, in triplicate. The corresponding volume of the working standard was evaporated under a stream of nitrogen and resuspended in 100 μL of blank plasma, kidney or liver homogenate by mixing in vortex for 2 min. Mycotoxins were then extracted as described in section 2.4 and then

analyzed. In order to assure the quantification of very high levels of OTA (levels in plasma higher than 600 μg/L could be expected), a dilution step was added when analyzing the recovery of 600 μg/L. Just after resuspending the plasma fortification extract in mobile phase (200 μL), 10 μL of this suspension were added to 190 μL of mobile phase, mixed and analyzed. The dilution factor was 20; therefore, it was possible to cover a range of up to 6000 μg/L in plasma. The recovery values (%) for all of the matrices were calculated by dividing the experimental mycotoxin concentration obtained by the nominal mycotoxin level. In the spiked plasma samples, the response was subtracted from the areas obtained in the plasma blank pool. The repeatability and intermediate precision of this process were studied by carrying out the complete recovery experiment for each matrix, for the 4 concentrations on one day and on three different days.

LOQ was determined by analyzing three replicates of fortified plasma (2, 4, 8 μg/L), kidney and liver (8, 16, 32 μg/kg). The lowest concentration for which acceptable data of recovery and precision was obtained was considered to be the LOQ (AEFI, 2001).

LOD was calculated theoretically using the method based on the calibration curve extrapolation at zero concentration (AEFI, 2001):

$$LOD = [Y_{bl} + (k \times S_{bl})] / b \qquad (k=3)$$

Where Y_{bl} and b are the intercept and the slope, respectively, of a curve that represents the area of each concentration versus the nominal concentration after analyzing the samples of spiked plasma, kidney or liver for obtaining the LOQ; k is 3; S_{bl} (standard deviation of the blank) is the intercept of the curve obtained by representing the standard deviation for each concentration level versus the nominal concentration.

Robustness: The influence of changes in the pH of the mobile phase (0.1% of formic acid in the aqueous phase), changes in the column batches (either new or in use), and the influence of column temperature (59ºC and 57ºC) on areas and retention times of a working standard of 30 μg/L of AFB1 and OTA were studied. Moreover, it was considered to be important to study the influence of light degradation of AFB1 during sample or standard manipulation (either in darkness or exposed to light). Three replicates of the working standard were analyzed in each condition and the concentrations obtained were compared with the nominal values (30 μg/L).

The acceptance/rejection criterions used were:

- Stability: peak areas ± 10% of the peak areas obtained just after sample preparation (0 h).
- Linearity: the determination coefficient (r^2) higher than 0.990, the slope interval not having to include zero ($p \leq 0.05$) and the intercept interval having to include zero ($p \leq 0.05$), the representation of residuals versus the estimated values having to rise to a distribution of the points at random and not having to reflect any trend, and the RSD (relative standard deviation) between response factors lower than 10%. ± 10% RSD and ±10% standard error of the mean (SE) for all the concentrations excepting the lower one, which is the limit of quantification, and whose accepted accuracy and precision was within ±15%.
- Recovery: ± 10% RSD in repeatability and intermediate precision conditions.
- Robustness: ±10% of standard error of the mean (SE).

Results and discussion

Up to now, many OTA determination studies have been performed on different biological samples and animal species using a wide variety of techniques (Ringot *et al.*, 2006; Vettorazzi *et al.*, 2008; Mantle, 2008; Zepnik *et al.*, 2003; Vettorazzi *et al.*, 2009; Zöllner and Mayer-Helm, 2006). However, there is a lack of modern techniques (LC/MS and HPLC) for studying the presence of AFB1 in body fluids or tissues, and most of the methods were initially limited to their use in food matrices (Herzallah, 2009; Siraj *et al.*, 1981), although later they were modified for their application to toxicological studies. Moreover, no validated UHPLC-FLD methods for simultaneous determination of OTA and AFB1 in plasma, kidney and liver have been previously described in laboratory animals, in spite of the fact that these methods would be of great interest for toxicological or toxicokinetic studies of mycotoxins.

Sample obtaining and mycotoxin extraction and cleanup.

For aflatoxins determination, chromatographic methods described by Siraj *et al.* (1981), Gregory (1982), Lamplugh (1983a,b) were not suitable for toxicological studies conducted with small animals. Coulombe and Sharma (1985) and Wong and Hsieh (1981) among others, used radioactivity quantification methods in order to achieve enough sensitivity and recovery for the tiny samples that are generated during toxicological studies. Plakas *et al.* (1991) combined HPLC quantification with

^{14}C-labeled AFB1 detection. In the case of OTA, Vettorazzi *et al.* (2008) validated a method successfully applied in a study of OTA toxicokinetics in rats (Vettorazzi *et al.*, 2009).

The sampling method has been inspired in the work described by Vettorazzi *et al.* (2008) for detecting OTA in plasma, liver and kidney of rats. OTA was extracted from the matrix with an organic solvent (ethanol) in very acid conditions using trichloroacetic acid (TCA). Unfortunately, this extraction process was not suitable for the simultaneous extraction of AFB1 and OTA because the pH conditions were too low for the AFB1 stability and because TCA, a trihaloacetic acid such as TFA (used for AFB1 derivatization (Lamplugh, 1983b; Kok, 1994), gives undesired chemical reactions. On the other hand, mycotoxins bind proteins (Newberne and Butler, 1969; Ringot *et al.*, 2006; Urrego Novoa and Díaz, 2006) and the extraction step needs low pH conditions to release OTA and AFB1 and to precipitate the proteins (Valenta, 1998). In addition, ACN obtained clearer extracts than ethanol in preliminary studies. Therefore, proteins from samples were precipitated with acidified ACN (1:3 ratio). Combinations of ACN-HCOOH (10%, pH 2.62 and 1%, pH 2.63), ACN-H_3PO_4 (1%, pH 2.72 and 0.5%, pH 2.84) and ACN-HCl (0.05%, pH 2.59) were assayed. After analysis of the resulting extracts, ACN-HCOOH 1% yielded clean enough extracts and recovery values over 90% for both mycotoxins in the three matrices.

With the procedure described, the simultaneous extraction of both mycotoxins has been achieved only starting from 100 μL of plasma or 25 mg of tissue and covering a range of 2-6000 μg/L in rat plasma and 8-2,400 μg/L in rat kidney and liver. Only one solvent step extraction is needed, avoiding immunoaffinity column clean up and toxic solvents such as chloroform, ethyl acetate or dichloromethane which cause health and environmental hazards.

Development of the UHPLC-FLD quantitative analytical method

Chromatographic conditions were investigated in order to achieve the best separation and resolution of peaks so as to allow quantification. The method starts with an isocratic set which permits the elution of the polar components extracted with the mycotoxins. Due to its polar properties, AFB1 appears early in the chromatogram. The front peaks corresponding to liver and kidney matrices are larger than those corresponding to plasma (figure 1). OTA needs an increase in the organic proportion of the eluent and it had to be delayed for up to minute 8.4 because several interfering peaks appear in kidney and liver, something that does not occur with plasma samples.

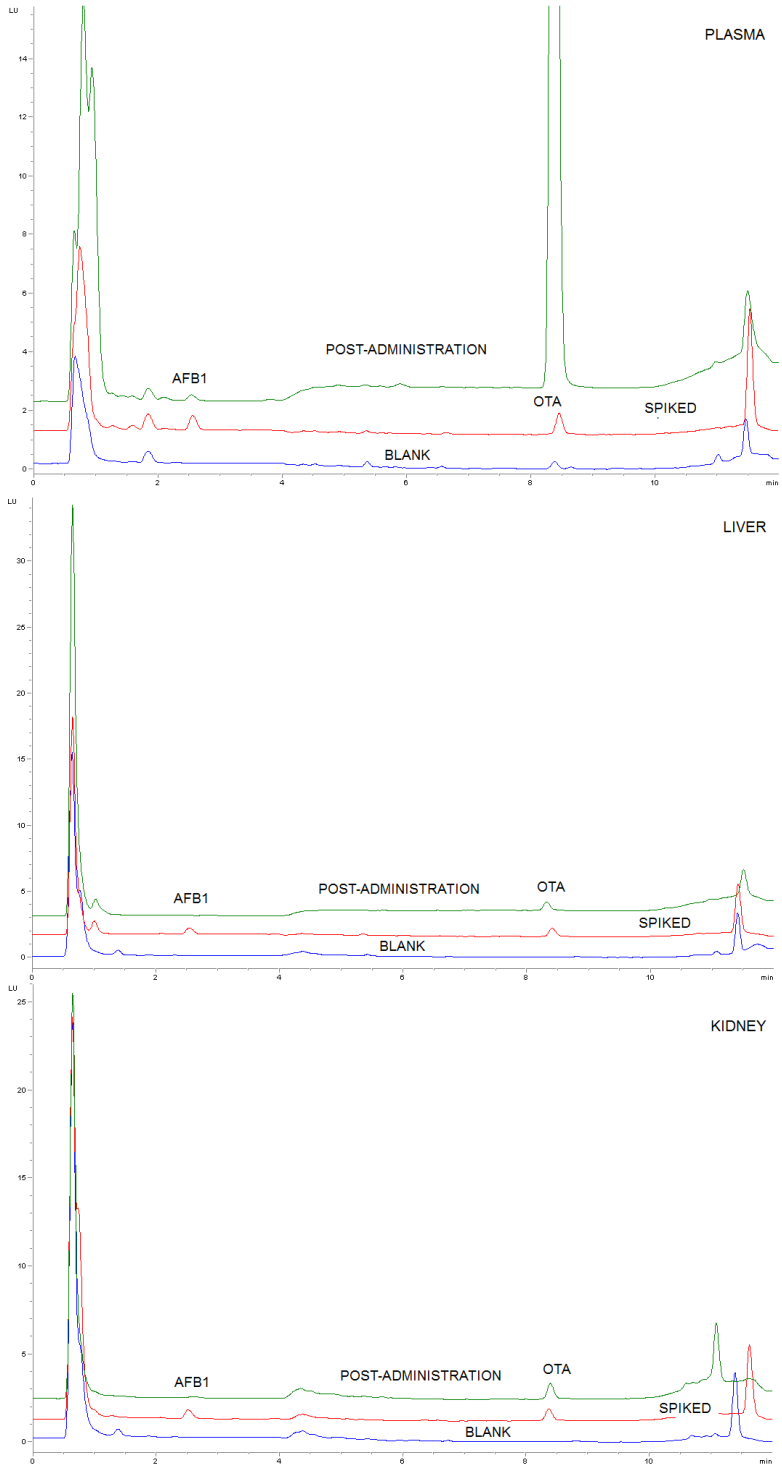

Figure 1: Superimposed chromatograms of blank, spiked and post-administration extracted sample of A) plasma, B) liver and C) kidney.

Aflatoxin B1 and ochratoxin A have fluorescent properties that make them good candidates for their detection with the use of fluorescence. However, it is well known than AFB1 suffers quenching in aqueous solvents; therefore, derivatization reactions would be necessary to quantify low levels. Either pre-column or post-column derivatization could be possible (Kok, 1994; Holcomb *et al.*, 1992; Joshua, 1993). Pre-column derivatization was initially discarded in order to obtain a simple and quick method. In addition, post-column iodine or bromine (Kobra cell) derivatization was also discarded because both reagents need to add extra pumps and chemical reactors to the UHPLC system. Furthermore, iodine derivatization decreases OTA peak intensity (Kok, 1994). The derivatization was performed with a photo-chemical reactor made with a knitted coil and a mercury lamp placed in line just after the column (Joshua, 1993). The PHRED makes derivatization fast and easy, and minimizes intervention of the analyst. The AFB1 signal is approximately 15 times more intense with the lamp on while OTA response remains unaffected.

Due to the fact that the same method has been applied for two mycotoxins in three different matrices, the effect of possible interferences of the matrix at the limit of quantification level has been studied for each one of them. There were no substantial differences in the chromatographic parameters for the same mycotoxin in the three matrices (see table 1).

Table 1: Chromatography parameters of AFB1 and OTA obtained at the concentration of the limit of quantification in plasma, liver or kidney.

	AFB1			**OTA**		
	Plasma	**Liver**	**Kidney**	**Plasma**	**Liver**	**Kidney**
Retention time (t_R) (min)	2.54	2.59	2.58	8.38	8.50	8.48
Retention factor (k')	2.45	2.95	3.05	10.38	11.97	12.29
Symmetry	0.77	0.78	0.65	0.94	1.09	0.97
Peak width at half height (w_h)	0.13	0.13	0.14	0.11	0.12	0.11
Number of theoretical plates (N)	2099	2115	1935	29631	28867	31130
Resolution (R_s)	3.11	7.61	9.26	21.41	20.58	20.58

Validation of the method

The method was selective for kidney and liver because no interference peaks appeared at the retention time of AFB1 or OTA in blank samples. In the case of plasma, no interference peaks appeared at the retention time of AFB1; however, basal OTA levels were detected in blank plasma. The experiments made in order to assure selectivity for OTA (reanalysis of spiked blank

contaminated plasma in different chromatographic conditions) showed that the standard OTA peak appeared at the same retention time as that of the interference. Moreover, after delaying of the peak, no broadening or distortion of the peak shapes was observed.

The working solutions remained stable up to 4 weeks at -20ºC (results not shown). After 6 weeks, the AFB1 concentration was unstable; after 8 weeks, the OTA concentration became unstable. Moreover, after 12 weeks, AFB1 started degrading in the working solution solvent (MeOH:ACN) while OTA remained unaffected. Spiked samples remained stable stored at -80ºC during 6 months (results not shown). This coincides with the stability observed for OTA in biological samples by Vettorazzi *et al.* in 2008. OTA and AFB1 were stable in processed plasma, liver and kidney for 15h in the HPLC tray at 4ºC without light (results not shown)

Linearity has been assessed in a wide range of concentrations in order to include not only high levels but also levels due to natural exposures that occur in laboratory animals fed with contaminated feed (Vettorazzi *et al.*, 2008; Mantle, 2008). The four calibration curves generated showed a good linear relationship between response (area of the peaks) and the respective AFB1 or OTA concentrations. All of the requirements for linearity have been met for the two concentration intervals of each mycotoxin (see table 2). Precision (RSD) and accuracy (SE) of the linearity showed adequate values, less than 10% in the whole interval (at the LOQ level less than 15%) (see table 3).

The recovery for each matrix was very efficient, between 93- 96% for AFB1 and between 94-96% for OTA. Moreover, the RSDs obtained in the within-day and between-day experiments were below 10% in each case (at the LOQ level below 15%), thereby demonstrating the precision of the analytical procedure (see table 4). The range of the recovery study went up to 600 μg/L in plasma, liver and kidney; however, according to Vettorazzi *et al.* (2008), higher concentrations of OTA might be expected in plasma, so the recovery of the dilution step was evaluated and the RSD was also below 10%. The recovery obtained in this method for AFB1 was successful for every matrix and higher than in other HPLC methods found in the literature (Siraj *et al.*, 1981; Lamplugh, 1983a; Coulombe and Sharma, 1985). In terms of OTA, the recovery was comparable to other methods for its analysis in plasma or serum, (Vettorazzi *et al.*, 2008; Zepnik *et al.*, 2003) or higher in liver and kidney (De Saeger *et al.*, 2004; Vettorazzi *et al.*, 2008).

For both mycotoxins, the LOQs were 2 μg/L in plasma and 8 μg/kg in liver and kidney and they were considered to be the lowest concentrations in their range of quantification (Bressolle *et al.*,

1996; ICH, 2005). The limits of quantification obtained for AFB1 using the photoderivatization instrument were satisfactory and better than others found in bibliography for HPLC methods (Gregory III *et al.*, 1983; Gregory, 1982; Lamplugh, 1983a; Lamplugh, 1983b). They were also comparable with radioactivity detection that uses very small amounts of sample (Coulombe and Sharma, 1985; Wong and Hsieh, 1980; Plakas *et al.*, 1991; Kok, 1994; Urrego Novoa and Díaz, 2006; Marzuki and Norred, 1984). For OTA, quantification limits are comparable to or better than those of other HPLC methods that use 2-100 times more sample volume (Valenta, 1998; De Saeger *et al.*, 2004; Vettorazzi *et al.*, 2008). The calculated LODs for AFB1 were as follows: 0.1 μg/L in plasma and 0.01 μg/kg in kidney and liver; the calculated LODs for OTA were: 0.3 μg/L in plasma and 0.01 μg/kg in kidney and liver.

Table 2: Linearity data calculated with three replicates of each concentration of the range

Mycotoxin	Range			Curve equation	Determination coefficient (r^2)	Slope limits	Intercept limits	RSD(%) response factor
	Standard (µg/L)	Plasma (µg/L)	Liver/Kidney (µg/kg)					
AFB1	0.5-30	2-120	8-480	y=2.11x+0.06	0.9988	(2.04; 2.18)	(-0.99; 1.10)	2.5
	30-150	120-600	480-2400	y=2.48x-7.78	0.9975	(2.31; 2.65)	(-23.72; 8.15)	6.1
OTA	0.5-30	2-120	8-480	y=2.52x+0.19	0.9989	(2.43; 2.60)	(-1.01; 1.38)	3.5
	30-150	120-600	480-2400	y=2.90x-7.45	0.9965	(2.66; 3.14)	(-29.56; 14.66)	6.3

Table 3: Precision and accuracy of the instrumental system

Mycotoxin	Concentration (µg/L)	Repeatability (within-day) (n=3)			Intermediate precision (between-day) (n=9)		
		Mean	RSD (%)	SE (%)	Mean	RSD (%)	SE (%)
AFB1	0.50	0.435	7.3	13.0	0.439	5.6	12.2
	2.0	2.10	4.1	5.3	1.98	5.7	1.0
	15	14.9	4.0	0.3	16.1	5.9	7.2
	30	31.7	6.4	5.8	30.0	6.0	0.0
	90	84.7	8.6	5.8	88.9	6.5	1.1
	150	147	2.1	1.7	153	3.8	2.4
OTA	0.50	0.450	8.6	9.9	0.446	7.8	10.8
	2.0	2.01	2.8	0.6	2.18	6.5	9.2
	15	14.5	3.5	3.6	15.7	6.3	4.6
	30	31.1	6.3	3.7	29.6	5.9	1.2
	90	87.1	8.3	3.2	91.2	6.9	1.4
	150	153	2.0	2.1	158	3.4	5.6

Table 4: Recovery study for AFB1 and OTA in plasma, liver and kidney

Mycotoxin		Concentration (µg/L)[a] (µg/kg)[b]	Repeatability (within-day)				Intermediate precision (between-day)			
			Recovery (%) n=3	RSD (%) n=3	Global recovery (%) n=12	RSD (%) n=12	Recovery (%) n=9	RSD (%) n=9	Global recovery (%) n=36	RSD (%) n=36
AFB1	Plasma	2	89.1	1.5	93.8	5.7	85.4	4.1	93.0	8.9
		8	95.1	2.6			97.1	5.5		
		120	101.5	1.6			102.9	2.1		
		600	89.7	1.0			86.7	5.0		
		(d=20)* 30	99.0	5.5			103.4	6.6		
	Liver	8	97.9	4.1	95.8	6.0	92.2	10.4	95.2	8.1
		32	94.8	2.5			97.8	5.2		
		480	101.9	2.9			102.5	2.3		
		2400	88.6	2.9			88.2	2.7		
	Kidney	8	94.2	3.3	97.0	5.8	100.7	6.2	96.0	8.1
		32	102.0	1.9			94.3	9.7		
		480	101.0	2.3			100.0	3.1		
		2400	91.2	6.5			88.7	5.4		
OTA	Plasma	2	97.9	1.5	96.5	7.5	92.8	5.8	96.0	8.9
		8	88.3	3.6			91.5	5.6		
		120	106.6	1.9			108.7	2,1		
		600	93.1	0.8			91.2	4.8		
		(d=20)* 30	96.1	5.8			103.2	7.9		
	Liver	8	82.5	4.9	91.3	10.0	90.3	8.7	94.4	8.8
		32	89.5	4.5			89.7	5.4		
		480	105.4	3.0			105.9	2.4		
		2400	90.3	2.8			91.5	2.8		
	Kidney	8	91.4	4.9	96.9	7.1	85.1	8.5	94.5	9.7
		32	95.4	5.7			91.6	5.6		
		480	105.6	2.0			106.1	2.5		
		2400	95.1	6.6			95.3	4.4		

[a] :µg/L for plasma, [b] :µg/kg for liver and kidney

*: recovery study of the dilution step (d=20) of plasma

In the study of robustness (see table 5), the pH of the mobile phase had a significant impact on the quantification of OTA. An increase in the pH yielded a decrease in the OTA signal, whereas AFB1 signal remains without changes. Ochratoxin A has been described as a weak acid and it is important to maintain a pH below 4.4 in order to assure that OTA molecule is in its protonated form (Valenta, 1998). In addition, different lots of columns were tested and the quantification was satisfactory while the retention times appeared to be affected. On the other hand, peak areas did not change after slight differences in column temperature. Due to the high pressure conditions of the chromatography, slight changes in temperature, column batches or flow affected the pressure; as a result, the retention times were affected but the quantification was considered to be robust. Moreover, it was very important to keep samples in the dark during their obtainment or manipulation due to the fact that AFB1 degrades easily when exposed to light.

Table 5: Study of robustness with standards: % of HCOOH in mobile phase, different column batches, changes in column temperatures and exposure to light.

	AFB1				OTA			
	Concentration (µg/L)		Retention time (min)		Concentration (µg/L)		Retention time(min)	
	Mean	SE (%)	Mean	SE (%)	Mean	SE (%)	Mean	SE (%)
Nominal	30		2.55		30		8.43	
0.1% HCOOH	30.0	0.0	2.53	0.7	25.7	14.2	8.33	1.1
Column batch A	29.4	2.0	2.21	13.0	30.6	2.0	7.48	11.3
Column batch B	27.7	7.5	2.38	6.3	30.6	1.9	7.81	7.3
Column at 59 ºC	29.0	3.2	2.55	0.0	30.0	0.1	8.33	1.2
Column at 57 ºC	29.3	2.2	2.65	4.1	30.1	0.3	8.69	3.1
Day light exposure	degraded				28.7	4.5	8.38	0.6

Application to real samples

The method was successfully applied in real samples obtained from rats that received administration of a mixture of AFB1 and OTA (0.5 mg/kg bw and 0.1 mg/kg bw, respectively) in a single dose by oral gavage (see table 6). The analytical results have been corrected with the recovery value for each mycotoxin. The highest level found in plasma for OTA was 919 µg/L, the dilution step (d=20) was applied and permitted quantification. On the contrary, low levels of AFB1 have been detected in plasma samples (<LOD). In kidney and liver, comparable levels of OTA have been found in both organs, and AFB1 was at a very low level in both of them.

Table 6: Concentration of AFB1 and OTA in plasma (μg/L), liver and kidney (μg/kg) from rats administered AFB1+OTA (0.5 mg/kg bw and 0.1 mg/kg bw, respectively) by oral gavage

		Concentration (μg/L)[a] (μg/kg)[b]	
	Sample	AFB1	OTA
Plasma	1	9.77	427
	2	3.06	919
	3	0.127[c]	543
	4	<LOD	528
	5	1.69[c]	549
	6	<LOD	422
	7	<LOD	424
	8	<LOD	420
Liver	1	<LOD	35.5
	2	<LOD	31.7
Kidney	1	6.06[c]	42.6
	2	6.58[c]	41.5

[a] :μg/L for plasma, [b] :μg/kg for liver and kidney

[c] :<LOQ

Conclusions

A rapid and simple method for the simultaneous quantification of AFB1 and OTA in rat plasma, liver and kidney by UHPLC-FLD has been validated and successfully applied. The process is economical because only low volumes of solvents are needed, and the use of immunoaffinity columns is not necessary in the purification process. In addition, this method uses a low-volume column that permits working under high pressure conditions, thereby giving high resolution in short time assays. The two most important advantages of this method are that it enables the simultaneous quantification of AFB1 and OTA in three biological matrices, and that only 100 μL of plasma or 25 mg of tissue are sufficient enough for obtaining results in a wide range of concentrations, with adequate recovery and LOD and LOQ values. These characteristics make it very useful for carrying out experimental work in toxicokinetic and toxicological studies.

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Chapter 6

An approach to the toxicity and toxicokinetics of aflatoxin B1 and ochratoxin A after simultaneous oral administration to F344 rats

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Resumen

Las micotoxinas son producidas por hongos y llegan al ser humano a través de alimentos contaminados. Algunas de las más peligrosas son la aflatoxina B1 (AFB1) y la ocratoxina A (OTA) que han sido clasificadas por la IARC como de clase 1 (carcinógeno humano) y de clase 2B (posible carcinógeno humano), respectivamente. Algunos autores han observado que tanto la AFB1 como la OTA pueden formar asociaciones sinérgicas *in vivo* con otras micotoxinas, por tanto sería interesante estudiar el comportamiento tóxico y toxicocinético de la mezcla de ellas.

Se ha realizado un estudio preliminar administrando una dosis única de 0,25 mg/kg pc de AFB1 y 0,5 mg/kg pc de OTA por vía oral a ratas F344. Se obtuvieron muestras de sangre 0.5, 2, 4, 6, 8, y 24 h después de la administración, y tras 8, 24, 48, 72 y 96 h los animales fueron sacrificados para obtener muestras de plasma, hígado, riñón, bazo y placas de Peier.

Los resultados bioquímicos e histopatológicos mostraron toxicidad aguda en el hígado provocada por la AFB1. No hubo toxicidad relevante en riñón ni órganos del sistema inmune. Según los resultados cinéticos, la absorción y metabolismo de la AFB1 son extremadamente rápidos. Solamente se encontró el 1% de la dosis inicial 10 minutos después de la administración, menos del 10% de la dosis en hígado tras 8 h, fue indetectable en riñón y los metabolitos desaparecieron del organismo tras 24 h. La concentración de OTA en plasma, hígado y riñón y algunos de los parámetros cinéticos son comparables con los resultados de Vettorazzi *et al.* (2010) bajo las mismas condiciones experimentales, por lo que parece que la AFB1 no afecta al perfil cinético de la OTA.

Abstract

Mycotoxins are produced by fungal species that reach human beings through contaminated food. Some of the most dangerous are aflatoxin B1 (AFB1) and ochratoxin A (OTA), classified by the IARC as class 1 (human carcinogen) and class 2B (possible human carcinogen), respectively. Some authors have found synergetic effects *in vivo* in the combination of AFB1 and OTA with other mycotoxins so it would be interesting to study their toxic and toxicokinetic behaviour together.

A preliminary toxicokinetic study with F344 rats has been carried out. Both AFB1 (0.25 mg/kg bw) and OTA (0.5 mg/kg bw) were administered together by oral gavage. Blood was extracted 0.5, 2, 4, 6, 8, and 24 h after administration. Furthermore, at 8, 24, 48, 72, 96 h the animals were sacrificed in order to determine mycotoxin concentration in plasma, kidney, liver, spleen and Peyer's patches.

The biochemical and histopathological results pointed to acute toxicity in liver due to AFB1. No remarkable toxicity was observed in kidneys or immunological organs. According to the kinetic results, the absorption of AFB1 was very fast and it metabolized rapidly. Only 1% of the dose was found in plasma after 10 min and less than 10% was observed in liver; it was undetectable in kidney and metabolites were undetectable after 24 h. The OTA concentration profiles in plasma, liver and kidney and some of the kinetic parameters are similar to those observed by Vettorazzi *et al.* (2010) under the same conditions as those used in this experiment; therefore, AFB1 did not change OTA kinetic profile.

Introduction

Mycotoxins are secondary metabolites produced by different fungal species that can be found in many agricultural commodities and processed food (Bennett and Klich, 2003). Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are some of the most relevant mycotoxins due to their toxic effects and demonstrated human exposure (EFSA, 2006; EFSA, 2007).

According to WHO, in the EU, daily exposure to aflatoxins is between 2 and 77 ng of AFB1 per person. The International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence in humans of the carcinogenicity of naturally occurring AFB1 (IARC, 1987; IARC, 1993); therefore, the authorities can not estimate a safe intake and advise the ALARA principle (as low as reasonable achievable) (EC, 2002). It has been described that, in humans, orally administered AFB1 follows a two-compartment model of absorption and elimination, with a rapid distribution phase followed by a slower elimination phase (EFSA, 2007). In rats, intestinal absorption of AFB1 is very fast and follows first-order kinetics (Ramos and Hernandez, 1996). Absorbed AFB1 reaches the liver through the portal system and is bioactivated by P450 cytochromes. The resulting epoxide attacks DNA-forming adducts (McLean and Dutton, 1995). AFB1 metabolism is well known (McLean and Dutton, 1995; El-Khatib *et al.*, 1998; Smela *et al.*, 2002; Bedard and Massey, 2006); on the contrary, data regarding kinetics of AFB1 in humans and in laboratory animals are sometimes contradictory or incomplete. In the majority of the studies, the authors used methods that could not detect the AFB1 molecule or differentiate it from the resulting metabolites (Wong and Hsieh, 1980; Coulombe Jr and Sharma, 1985; Cupid *et al.*, 2004; Jubert *et al.*, 2009; Firmin *et al.*, 2010).

With regard to OTA, this mycotoxin is a potent nephrocarcinogenic compound in rodents and, despite the controversy, it is considered to be an indirect genotoxic agent (Arbillaga *et al.*, 2004; Arbillaga *et al.*, 2007; Mally *et al.*, 2004; Mally *et al.*, 2005; Mally and Dekant, 2005; Turesky, 2005). The IARC classified OTA as a possible carcinogenic compound (IARC, 1987; IARC, 2002). OTA mechanisms of action are not clearly determined but apparently, its ability to generate reactive oxygen species (ROS) may explain the lipid, protein and DNA damage (Ringot *et al.*, 2006). Different official organizations have suggested a safe PTDI (provisional tolerable daily intake) between 5 and 17 ng of OTA per kg of body weight and the levels found in plasma in humans in different countries ranges between 0.01 and 75 μg/L (Coronel *et al.*, 2010). In most animal species, the kinetic behavior of OTA has been described as a two compartment open model, although recent data regarding the

accumulation in kidneys suggest that these models are too simple and should be re-analyzed using multi-compartment models (EFSA, 2006). Upon absorption from the gastrointestinal tract, OTA binds to serum proteins (approximately 99%). Considerable variations in serum half-lives across species have been reported due to the affinity and degree of protein binding (O'Brien and Dietrich, 2005). Reabsorption of OTA from the intestine back to the circulation, as a consequence of biliary recycling, favors the systemic redistribution of OTA towards the different tissues. In addition, reabsorption of OTA occurs in the kidney proximal and distal tubules which enhance accumulation in blood, liver and kidney and explains the long life of OTA in plasma and tissues (Ringot *et al.*, 2006). Vettorazzi *et al.* have performed a series of kinetic studies and reported that sex, gender and fasting conditions may have an impact on the kinetic profile of OTA (Vettorazzi *et al.*, 2009; Vettorazzi *et al.*, 2010; Vettorazzi *et al.*, 2011).

Human and animal population is exposed to multiple mycotoxins because the same food might be contaminated by more than one mycotoxin, and mycotoxins might reach humans from different sources. Co-exposure to different mycotoxins, could originate synergic or additive toxic effects on human or animal health; however, knowledge regarding this aspect or regarding the influence of co-ocurrence on toxicokinetic or toxicological characteristics of the mycotoxins is still scarce. There are toxicological studies using mixtures of AFB1 and OTA in swine, rabbits, rats, etc. and the results show synergetic, additive or antagonic associations depending on the endpoint analyzed (Huff and Doerr, 1981; Huff *et al.*, 1983; Wangikar *et al.*, 2004; Wangikar *et al.*, 2005). In a large study combining AFB1 and OTA, the mixture showed no interaction regarding the measurement of mortality, weight gain and most serum biological parameters but the anaplasic and hiperchromatic nuclei, necrosis and bile duct proliferation observed were more pronounced in the combined toxin group after 4 months (Rati *et al.*, 1981). However, multi-exposure to mycotoxins has not been taken into account in any of the kinetic studies published to date.

The main objective of this work was to study the kinetic behavior of AFB1 and OTA after a single oral dose of both mycotoxins in rats, in order to investigate possible changes in their pharmacokinetic profile in relation to those reported in previous studies, due to the presence of both mycotoxins; another objective was to determine whether or not this kinetic behavior could have a role in their interaction. Moreover, toxicity parameters were studied so as to obtain a more ample approach to the mixture interaction (ICH, 1994).

Material and methods

Safety precautions

Aflatoxin B1 and ochratoxin A are toxic substances. They were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all procedures carried out and during the manipulation of treated animals or contaminated samples, FPP3 masks were used.

Chemicals and reagents

For administration to the animals, AFB1 and OTA were purchased in powder from Sigma (Steinheim, Germany). AFB1 was dissolved in DMSO to an initial concentration of 4.73 g/L and OTA was dissolved in 0.10 M $NaHCO_3$ (pH 7.4) (Riedel-deHaën, Seelze, Germany) to an initial concentration of 1.00 g/L. All the solutions were maintained at -20ºC until use. Aflatoxin B1 was kept in the dark to avoid degradation. The mixture of AFB1 and OTA that was administered (0.025 g/L and 0.050 g/L, respectively) was prepared from the initially concentrated forms.

For the analytical quantification of mycotoxins, AFB1 was purchased as a solution of 2 mg/L in acetonitrile (ACN) and OTA was purchased as a solution of 10 mg/L in ACN, both from OEKANAL® Fluka (Schnelldorf, Germany) as certified reference materials. For the tissue homogenates, $NaH_2PO_4.H_2O$ (Merck, Darmstadt, Germany) at 0.05 M, pH 6.50, was used. All the reagents used for the HPLC analysis were of analytical grade. ACN and methanol (MeOH) HPLC grade and formic acid were obtained from Sigma Aldrich (St. Quentin Fallavier, France).

Animals

All the animals used, ten-week-old male Fisher 344 (F344) rats, were purchased from Harlan (Horst, The Netherlands). On the day of arrival, the animals were weighed (weight variation did not exceed ± 20% (OECD, 1984; OECD, 2009) and then distributed into polycarbonate cages with stainless steel covers for one week in order to allow acclimatization to the environmental conditions: 12 h day/night cycle, temperature 22 ± 2ºC, relative humidity 55 ± 10%, standard diet (Harlan Iberica, Spain) and water *ad libitum*. The *in vivo* experiments were approved by the Ethics Committee on Animal Experimentation of the Universidad of Navarra.

Study design and sample collection

The animals were randomly distributed into 5 groups of 3 animals per group. After at least five days of acclimatization, the animals received oral administration of a single dose of a mixture of 0.25 mg/kg bw of AFB1 and 0.5 mg/kg of OTA in $NaHCO_3 \cdot H_2O$ (0.1M pH 7.4). The volumes of administration were 10 mL/kg bw; therefore, the volume and dose administered were adjusted to the animal weight. A control group with two rats was added in order to obtain control samples and assure that no cross-contaminations occurred during the study. They received oral administration of a mixture of NaH_2PO_4 x H_2O (0.1 M pH 7.4) with 0.5% DMSO.

In order to determine the AFB1 and OTA concentrations in plasma at 10, 30 min, 2 and 4 h, blood from 3 animals per time point was collected from the retro-orbital sinus under isoflurane anesthesia. At 8, 24, 48, 72 and 96 h, plasma was obtained by decapitation (n=3 per endpoint). Blood was extracted from each animal only once before sacrificing; extraction time points for each rat were chosen taking into account the time for volemia recovery (Diehl *et al.*, 2001). After retro-orbital extraction or decapitation, blood was collected into heparinized tubes (BD Vacutainer system) for clinical biochemistry analysis and AFB1 and OTA determination. Blood samples were centrifuged (1,085 × g for 15 min at 4°C) in order to obtain plasma, which was stored at −80°C.

The livers and kidneys were extracted from the animals, washed with water until the external blood was removed, blotted on filter paper, and finally weighed. Kidneys were sliced longitudinally into two halves and the liver was cut into five pieces. One half of each kidney and a piece of the biggest lobe of each liver, one half of spleen and some Peyer´s patches were fixed in 4% formaldehyde solution, dehydrated and embedded in paraffin for histopathological analysis. The other three halves of kidney and the rest of the liver pieces were packed individually, flash-frozen in liquid N_2 and stored at -80°C for mycotoxin determination. In order to prevent cross contamination between samples, all the dissection material was cleaned with water and rinsed with ethanol after each animal necropsy.

Clinical biochemistry and histopathology

In order to analyze possible weight changes in the target organs due to the administration of the mycotoxins, the relative weight (RW) of liver and kidneys were calculated dividing the weight of each organ by the total weight of the animal.

Biochemical analyses of plasma samples were performed with a Hitachi 911™ (Roche Diagnostics) analyzer using the protocols obtained from Roche for the determination of the standard parameters in plasma: total protein (g/dL), albumin (g/dL), glucose (mg/dL), aspartate transaminase (AST) (U/L), alanine transaminase (ALT) (U/L), alkaline phosphatase (U/L) and urea (mg/dL).

For the histopathological examination, paraffin sections (3 μm) were cut, mounted onto glass slides, and dewaxed and stained with hematoxylin and eosin (H&E). In the observation and evaluation of each sample, the systemic anatomopathological protocol was applied, with special attention to:

- Normalcy or alteration of the architecture and proportions of the cutaneous structures.
- Presence of circulatory phenomena.
- Evaluation and quantification of degenerative or necrotic phenomena.
- Existence or absence of inflammatory phenomena, types and intensity.
- Abnormal growths: atrophy, hyperplasia, hypertrophy, neoplasia.
- Particular or special findings.

The evaluation of some of these alterations (circulatory, degeneration and/or necrosis, inflammation and growth abnormalities) was carried out by calculating the different "fields" with the adequate increases for their correct observation, using a scale from 0 to 5 in order to express the results obtained. Whenever necessary, measurements are taken by means of the calibrated digital system.

Determination of mycotoxins in plasma, liver and kidney

The concentration of mycotoxins in plasma and tissues was determined by UHPLC with fluorescence detection. The extraction procedure and the UHPLC-FLD quantification method was previously set up and validated for these biological samples (Corcuera *et al.*, 2011). Kidney and liver were homogenized in a round-bottom plastic tube with 4μL of cold sodium phosphate buffer (0.05 M, pH 6.50) per mg of tissue. The tissue homogenates were aliquoted and stored for at least one day at -80ºC until the mycotoxin extraction was carried out. The plasma samples or the tissue homogenates were kept at room temperature for 30 min before the extraction step. Next, 100 μL of plasma or tissue homogenate were treated with 300 μL of the extractive solution (ACN acidified with

formic acid 1%) which precipitated the proteins and released the mycotoxins. After mixing it in a vortex, they were centrifuged at 6200 x g for 15 min at 4ºC in order to separate the protein fraction from the supernatant that contained the mycotoxins. The supernatant (200 μL) was evaporated and the solid residue was resuspended in 200 μL of: H_2O (1% HCOOH): MeOH:ACN 50:50 (0,1%HCOOH), 60:40.

Analyses were performed on a total of 63 samples (29 plasma, 17 liver and 17 kidney) in an Agilent Technologies 1200 liquid chromatographic system equipped with a fluorescence detector (G1321A model) controlled by ChemStation B.03.02 software (Hewlett-Packard). Mycotoxins were separated on an Ascentis® Express C18 column (150 mm x 2.1 mm; 2.7 μm) from Supelco (PA, USA). The injection volume was 40 μL and the flow rate was 0.9 mL/min. Chromatography was performed at 60ºC. The mobile phase was a mixture of an organic phase (A) (MeOH-ACN, 50:50, v/v) and water (B), both acidified with 0.5% of formic acid. Proportions of both organic and aqueous phases were switched between isocratic and gradient profiles during the entire analysis procedure. The elution program starts with the isocratic profile until minute 2.4 with 30% of A, then from minute 2.4 to 2.5 the organic phase increases up to 43%, from minute 2.5 to 8.3 min another isocratic profile at 43% of A, from minute 8.3 to 10.0 there is a last increase up to 65% of A and finally, from minute 10.0 the system returns to 30% of A to restore the starting conditions during 5 min. The retention times under these conditions were 2.5 min for AFB1 and 8.4 min for OTA. Before the sample entered the fluorescence detection cell, and in order to increase sensitivity for AFB1, a photoderivatization device (AURA Industries, NY, USA) with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.25 mL (5 m x 0.25 mm) was included. During the first 4 min of analysis, fluorescence conditions were optimized for AFB1 (excitation 366 nm and emission 433 nm wavelengths), and after that, for OTA (excitation 225 nm and emission 461 nm wavelengths).

The LOQ were 2 μg/L in plasma and 8 μg/kg in liver and kidney for both mycotoxins. The LODs for AFB1 were as follows: 0.1 μg/L in plasma and 0.01 μg/kg in kidney and liver; the LODs for OTA were: 0.3 μg/L in plasma and 0.01 μg/kg in kidney and liver. Recovery was very efficient for both mycotoxins in plasma and tissues (between 93 - 96% for AFB1 and between 94 - 96% for OTA), and the relative standard deviation (RSD) obtained within and between day experiments was below 10% in all the matrices studied. All the mycotoxin levels obtained have been corrected by the recovery value for each matrix.

Statistical analysis

Data are presented by descriptive analysis as mean ± standard deviation (SD) of three animals. The distribution of the data was checked for normality using the Shapiro-Wilks test. The homogeneity of the variance was verified by the Leven test. The comparisons were performed using the Kruskal-Wallis test followed by DMS test. P-values equal or below 0.05 were accepted as the level of significance.

Results

Clinical biochemistry and histopathology

The treated animals did not show clinical signs of toxicity during the experiment. However, during the necropsies, after 48, 72 and 96 h, the livers of the treated animals were light red with a visible loss of color in comparison to the control animals. Moreover, in treated animals, the relative weight of the livers after 48, 72 and 96 h was significantly higher than in the control animals (table 1).

The biochemical parameters of the treated animals were comparable to the control values with the exception of the transaminases: ALT and AST (table 2). The increases in ALT and AST are signs of hepatocyte death due to hypoxia, fatty change or necrosis (Smith *et al.*, 2002). Their values increased after administration of the mycotoxins, and AST reached a maximum after 48 h, while ALT reached the maximum after 72 h. Afterwards, the levels returned to control levels, showing a recovery of the liver after acute damage.

The most evident alterations were detected in liver while the renal alterations are the least significant. The lymphoid organs represented by the spleen, thymus and Peyer's patches showed no differences between the control and treated groups at the different observation times.

In the liver, a progressive lesional state was observed. It is characterized as follows: at 8 h diffuse hepatocyte necrosis, accompanied by focal hepatitis, stands out. At 24 h hepatocyte necrosis continues to be observed in porta spaces and parenchyma (figure 1 B). At the same time, discreet degeneration begins to be observed in the cytoplasm of the hepatocytes and a few of these appear to be binucleated or with large nuclei. At 48 h a large number of hepatocytes present intense tumefaction and degeneration in the cytoplasms (figure 1 C), while others show necrosis surrounded by inflammatory infiltrates. In the porta spaces, they proliferate with bile canaliculi and a process of fibrosis begins (figure 1 C). At 72 h the diffuse hepatocyte necrosis and the inflammatory response in the parenchyma are maintained. Degeneration of the hepatocytes in the cytoplasm is scarcely

evident. The fibrosis and proliferation of bile canaliculi in the porta spaces as well as focal fibrosis of the parenchyma are notable (figure 1 D). This fibrosis is interpreted as small areas of cicatrization due to the loss of hepatocytes. Together, these findings can be defined as the very initial stages of cirrhosis. At 96 h hepatocyte necrosis is no longer observed and the inflammatory response is either stabilized or has decreased. The cytoplasm of the hepatocytes presents tumefaction and moderate degeneration. Proliferation of bile canaliculi and portal fibrosis are largely present (figure 1 E) as well as regenerative phenomena in the hepatocytes with numerous binucleated cells (figure 1 F).

In the kidney, the proximal convoluted tubules progressively lost the renal glucogen. At the same time, in the glomerules, hypercellularity was observed due to the infiltration of inflammatory cells, resulting in glomerulonephritis. Over time, sclerosis and moderate glomerular atrophy were observed. The alterations in the renal tubules were not evident and even though there was an increase of interstitial nephritis and interstitial fibrosis observed as of 48 h, no previous serious tubular lesion was found.

Plasma and tissue mycotoxin concentrations

After administration of mycotoxins, levels of OTA were found in plasma and tissues at all the timepoints, and very low levels of OTA were detected in control samples (<LOQ), presumably due to OTA contamination of standard diet of animals (Vettorazzi *et al.*, 2009; Zepnik *et al.*, 2003; Mantle, 2008). The amount in plasma increased as of 10 min and reached a maximum at 2h (C_{max} obs = 4,326.6 μg/L) and afterwards, it slowly decreased, with a plasma half live of approximately 48 h (figure 2A). The plasma concentration that Vettorazzi *et al.* (2010) obtained in a previous work with adult male F344 rats in similar experimental conditions has been overlapped in chart A in order to compare the kinetic curve with the data from this work. In tissues, the OTA concentration was similar in liver and kidney, reaching maximum levels at 8 h (figure 2B).

AFB1 could only be quantified in plasma at 10 and 30 min. In liver, levels below the LOQ were observed 8 h after administration, and no AFB1 was detected in kidney. In plasma, chromatograms showed a wide front peak, very different from the control or AFB1 spiked plasma samples. This indicated the presence of new compounds with more hydrophilic properties than AFB1 or OTA. In order to enlarge the front peak, chromatographic conditions were modulated and at least 8 different peaks appeared (figure 3). They had maximums at 30 min and then decreased in a time-dependant manner until 24 h. After 48 h, no peaks appeared in the front.

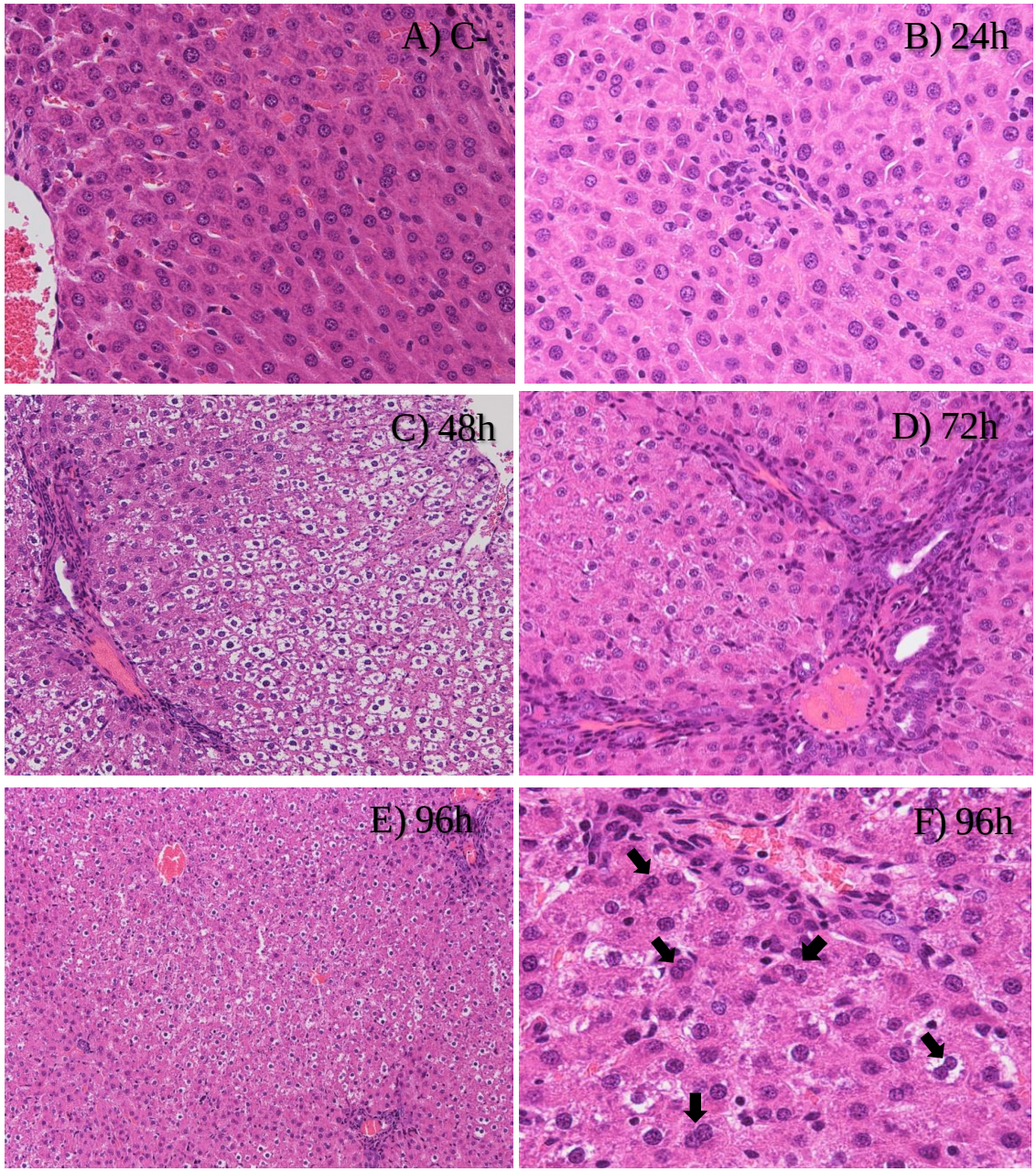

Figure 1: Microscopic images of liver samples. A progressive lesional state can be observed. A) Normal liver of a control sample. B) Focal cell necrosis and discrete degeneration of cytoplasm after 24 h. C) Intense tumefaction of hepatocytes, cell proliferation and initial fibrosis in porta spaces after 24 h. D) Necrosis and inflammatory response maintained fibrosis around bile canaliculi in porta spaces after 72 h. E) Moderate tumefaction of cytoplasms, proliferation of bile canaliculy and portal fibrosis after 96 h, and also F) binucleated hepatocytes in a regenerative fenomena.

Table 1: Mean relative weight of the liver and transaminases (AST and ALT) levels of each time group. The values of treated animals have been compared with those of the control group ($p \leq 0.05$*). Data are presented as mean±SD (n=3).*

	Relative liver weight	AST (U/L)	ALT (U/L)
Control	3.03 ± 0.04	115 ± 4	71 ± 6
8h	3.06 ± 0.11	212 ± 117	120 ± 18
24h	2.81 ± 0.11	282 ± 87*	213 ± 91*
48h	4.13 ± 0.07*	642 ± 361*	544 ± 314*
72h	3.62 ± 0.15*	549 ± 141*	573 ± 175*
96h	4.25 ± 0.47*	215 ± 128	139 ± 62

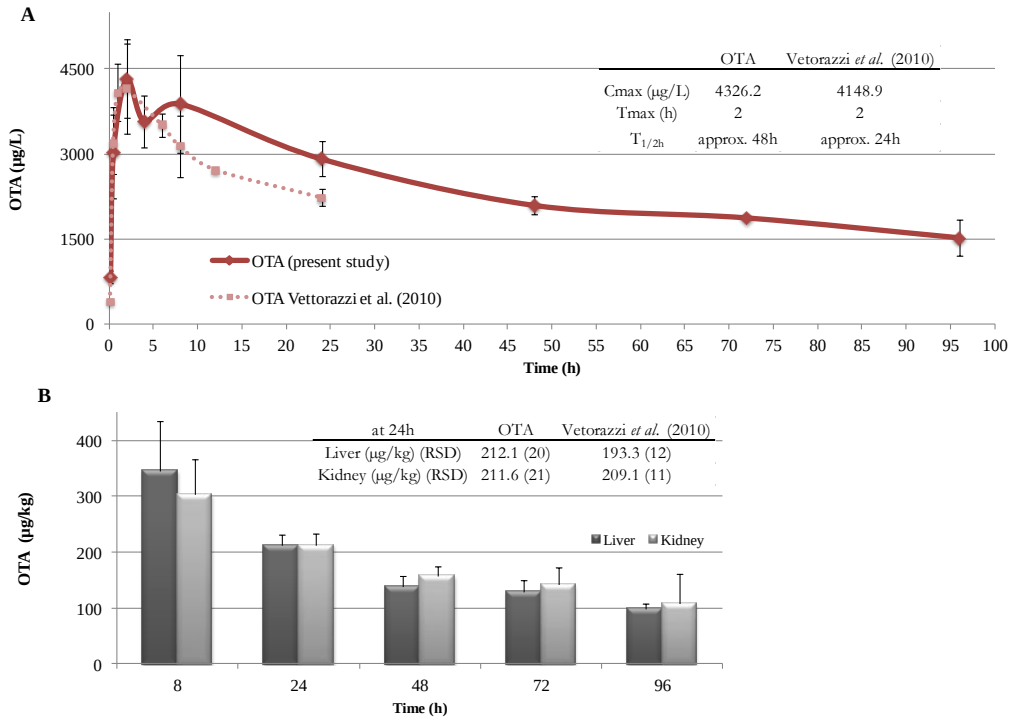

Figure 2: Plasmatic (A) or tissue (B) concentrations of OTA overtime after a single oral administration of AFB1+OTA (0.25 mg/kg bw of AFB1 and 0.5 mg/kg of OTA in $NaHCO_3 \cdot H_2O$ *(0.1M pH 7.4)). The plasma and tissue values that Vettorazzi et al. obtained in 2010 under the same experimental conditions have been included. Also, the kinetic parameters (*C_{max}*,* T_{max} *and* $T_{1/2h}$*) of the present experiment and the Vettorazzi et al. have been superimposed on A graph.*

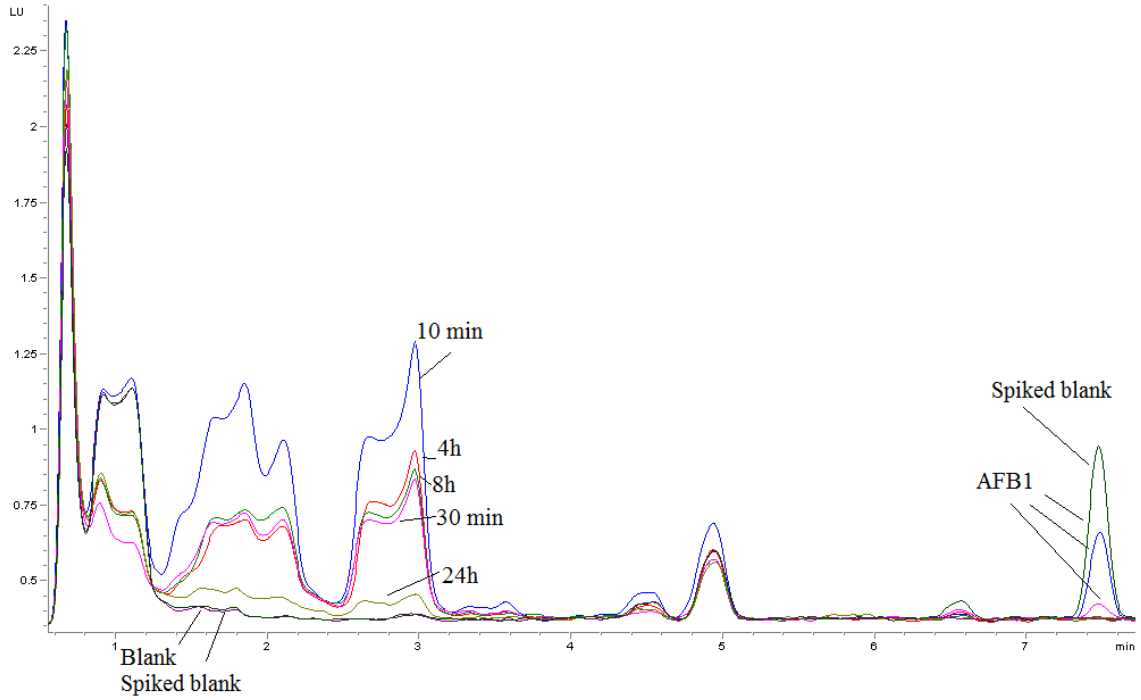

Figure 3: Superimposed chromatograms of plasma samples collected 10 and 30 min, 4, 8 and 24 h after administration. Chromatograms of a blank plasma sample and a spiked plasma sample with AFB1 and OTA have been included.

Discussion

The ICH guideline S3A highlights the need to integrate pharmacokinetics into toxicity testing, which should aid in the interpretation of the toxicology findings (ICH, 1994). With this aim, our study attempted to learn more about the toxic and kinetic behavior of the mycotoxins AFB1 and OTA when they are administered together. Wong and Hsieh (1980) described that rats are one of the most sensitive species to AFB1 acute toxicity and carcinogenic effects. OTA has been described as a potent nephrocarcinogen in male rodents and its kinetic profile depends on sex, gender and fasting conditions (Vettorazzi *et al.*, 2010; Vettorazzi *et al.*, 2011). Due to the aforementioned, young male rats were selected for this experiment. To avoid the interaction of food, the mycotoxins were administered in fasting conditions. In rats, doses from 0.2 to 12.5 mg/kg bw were used in single or repeated oral dose toxicity studies of AFB1 toxicity (Wong and Hsieh, 1980; Coulombe Jr and Sharma, 1985; Rati *et al.*, 1981; Bannasch *et al.*, 1985; Raj *et al.*, 1998; Ellinger-Ziegelbauer *et al.*, 2006; Theumer *et al.*, 2010). A low dose of AFB1 (0.25 mg/kg bw) which corresponds to approximately

3% of LD_{50} (Wong and Hsieh, 1980; EFSA, 2007) was administered in order to avoid strong acute toxicity of AFB1. The single OTA dose selected (0.5 mg/kg bw) was approximately the carcinogenic dose described by the NTP studies during 24 months (0.7 mg/kg bw). It corresponds to 2.5% of the LD_{50} (NTP, 1989), it has been used in recent studies (Zepnik *et al.*, 2003), and it was the same as that used by Vettorazzi *et al.* (2009); therefore, some comparisons could be made.

Although there were no signs of general toxicity during the experiment, the biochemical and histopathological results pointed to acute toxicity in liver and no toxicity in kidney or other organs. The signs of hepatotoxicity appeared 48 h after administration, with paleness of livers, increases in transaminases, cell necrosis and inflammatory infiltration. However, the liver started a repairing process after 72 h and its effects were evident after 96 h because AST and ALT almost returned to normal values. At that time, the regenerative phenomena were evident by the numerous binucleated cells present. These findings coincide with the observations made by of Rati *et al.* (1981) regarding acute toxicity of AFB1, and it appears that the AFB1 and/or its toxic metabolites are eliminated (metabolized and excreted) in a range of 48-96 h. No remarkable toxicity was observed in kidneys or immunological organs (spleen, thymus and Peyer's patches) so OTA did not cause acute toxicity at this dose, as expected. In conclusion, the acute toxic effects observed after the combined treatment of AFB1 and OTA were mostly due to AFB1 effect.

According to the evident signs of toxicity in liver, high AFB1 levels might have been expected in plasma and liver at least at the early time points of collection, and detectable levels in plasma during at least 72 h. Surprisingly, only an astonishing 1% of the administered dose was found in plasma after 10 min and 0.4% after 30 min. The liver levels were lower than the LOQ (8 μg/kg) at all time points and AFB1 was undetectable in kidney. For this reason, it was not possible to calculate any kinetic parameter. These findings suggest that the absorption of AFB1 was very fast, and that the molecule was rapidly metabolized in the liver. Moreover, almost nothing of the un-metabolized AFB1 was distributed outside the liver in plasma to kidneys. The compounds detected in the front peak are assumed to be AFB1 metabolites because they have not been detected in spiked plasma sample with AFB1 and OTA nor in recent OTA kinetic studies (Vettorazzi *et al.*, 2009; Vettorazzi *et al.*, 2011; Vettorazzi *et al.*, 2007). These compounds had a maximum at 30 min, decreased in a time-dependent manner until 24 h, after which they were undetectable. Coulombe and Sharma (1985) described a two compartmental model for AFB1 kinetics in rats, reaching the maximum concentration after 3 h and with the plasma half life being 91.8 h. A more recent study found the

maximum concentration 4 h after administration, with plasma half live of 53 h (Firmin *et al.*, 2010). This data describes long plasma half-lives, with long elimination phases after quantifying only AFB1 or mixed with its metabolites. These observations do not coincide with our findings in which AFB1 disappeared from plasma in 30 min, and its metabolites in 24 h, and this suggested an extremely fast uptake and metabolism of AFB1. Jubert *et al.* (2009) observed a rapid uptake (t_{max} = 1 h) in humans, with urinary elimination of 95% of the dose in 24 h, which is closer to our findings; in their work, they could not differentiate among AFB1 and its metabolites. It could be possible that OTA modifies/accelerates AFB1 metabolism in liver into very hydrophilic compounds that are rapidly excreted (24 h) through the kidney.

With regard to OTA, elimination occurs mainly from the liver compartment (Vettorazzi *et al.*, 2011), and as AFB1 clearly affects liver, it was expected that OTA profile in plasma and/or tissues may differ if it was co-administered with AFB1. The OTA concentration profiles in plasma, liver and kidney and some of the kinetic parameters are comparable to the ones that Vettorazzi *et al.* obtained under the same conditions of this experiment (figure 2A) but only with OTA. The maximum concentration in plasma was obtained after 2 h and corresponded to 87% of the initial dose, which was similar to the 83% that Vettorazzi *et al.* (2010) reported. However, an apparent delay in the elimination of OTA resulting in a higher plasma half-life has been observed. This event might be explained because the liver had suffered acute damage which had affected its function in the metabolism of OTA. In this case, higher doses of OTA (in comparison with OTA alone) would have been expected in liver and kidney. However, between OTA alone and OTA administered with AFB1, no significant differences were observed in the tissues after 24 h. Comparing the OTA obtained profile with other experiments and models of OTA kinetics, the differences in the elimination of OTA are more closely related to the age and sex of the rats than to the presence of AFB1 in the system (Vettorazzi *et al.*, 2007; Vettorazzi *et al.*, 2010; Vettorazzi *et al.*, 2011).

In conclusion, more specific information was obtained regarding the behavior of mycotoxins AFB1 and OTA in F344 rats. The acute toxic effects observed in liver were mostly due to AFB1, and this confirms that AFB1 was absorbed rapidly. A validated UHPLC-FLD method was used to selectively analyze AFB1 and OTA in plasma and tissue samples. AFB1 was rapidly metabolized and eliminated in 24 h but it was not possible to determine if OTA affects AFB1 uptake and metabolism because there was not enough data available regarding AFB1 kinetics under similar experimental conditions. With regard to OTA, it appeared that its levels in plasma and tissues and its toxicity were

not affected by the presence of AFB1, but its elimination could be affected. However, further investigations should be carried out in order to confirm this hypothesis.

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Chapter 7

Ochratoxin A reduces Aflatoxin B1 genotoxicity: Simultaneous application of the *in vivo* micronucleus and comet assays

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In preparation

Chapter 8

General discussion

Aflatoxin B1 and ochratoxin A are two of the most prevalent mycotoxins that can be found in edible products. Aflatoxin B1 has been classified as a human carcinogen; its mechanism of action, as a direct genotoxin, has been extensively studied. Ochratoxin A is a potent carcinogen in rodents but the evidence in humans in not sufficient, and the genotoxic routes are not elucidated yet. According to the literature, more than one mycotoxin may occur together in the same food, human diet is varied and furthermore, the manufacturing process mixes together different raw materials, yielding totally new matrixes with a new risk profile (Binder *et al.*, 2007). Due to the fact that humans and animals are exposed to a mixture of mycotoxins and the toxic effects of the possible combined associations are still unknown, the combined toxicity needs to be studied.

In this general discussion, the most relevant results found in this research project regarding AFB1 and OTA combined genotoxicity will be summarized as follows:

In vitro genotoxicity and oxidative damage in Hep G2

With the aim of studying the possible genotoxic effects, the comet assay complemented with restriction enzymes was carried out in human liver cells. Hep G2 cell line retains some phase I and phase II enzymes (Knasmuller *et al.*, 1998) and it has been proven to be useful for detecting genotoxicity with the comet assay after prolonged treatments with promutagens (Uhl *et al.*, 1999). The comet assay has still not been validated and there are no recommended guidelines. For this reason, recommendations regarding mutagenicity assays on mammalian cells have been taken into account (OECD, 1997a; OECD, 2010). In order to establish the adequate dose range, cytotoxicity assays were previously performed at 3 h and 24 h under the same conditions. Doses with survival values under 60% were not used in the genotoxicity tests. The comet assay was performed at the short time of 3 h with and without external metabolic activation in order to avoid cell reparation of the possible DNA damage as the guidelines suggest, but also at 24 h in order to take advantage of the inner metabolic competence of Hep G2.

Aflatoxin B1 resulted more cytotoxic than OTA in Hep G2 cells, and DNA strand breaks were detected after 3 h with metabolic activation and after 24 h without metabolic activation. In fact, the inner metabolic competence of Hep G2 revealed more genotoxic events than the external metabolic activation. The comet assay was able to detect the different types of DNA damage caused by AFB1 after its bioactivation (Bedard and Massey, 2006): the reaction of AFB1-exo-8,9-epoxide with guanine gives the adduct AFB1-N7-Gua, the AP site formed after the loss of adduct with

guanine, and the breaks in the repairing process were detected as direct strand breaks; and the lesion specific restriction enzyme FPG was able to detect the formamidopyrimidines formed after the base-catalyzed hydrolysis, where the imidazole ring of the adduct opens to an AFB1-FAPY (Smela *et al.*, 2002; Collins *et al.*, 2008). The dichlorofluorescein assay was performed after 24 h when the oxidative damage was clearly observed. A low, but significant, increase in intracellular ROS was detected; therefore, it is presumed that the oxidative secondary route may play a role in AFB1 genotoxic action (Bedard and Massey, 2006).

No genotoxic effects were observed after 3 h or 24 h with OTA in the concentration range, being clearly cytotoxic after 24 h. These results do not coincide with those reported by other authors (Ehrlich *et al.*, 2002; Simarro Doorten *et al.*, 2006; Arbillaga *et al.*, 2007a), who found mild genotoxic effects, but only at cytotoxic concentrations which also showed intracellular ROS (Arbillaga *et al.*, 2007a). Clear but discrete ROS increases were found in the range tested at 24 h, possibly due to OTA reactivity with GSH and quinones yielding ROS-acting forms as secondary products (Ringot *et al.*, 2006). Since Hep G2 cells retain metabolic competence, they might be able to detoxify OTA to the less toxic metabolites and consequently, reduce ROS formation and DNA oxidation.

In combined 24 h treatments, the cytotoxicity observed was more pronounced than in single treatments, and the results are consistent with an additive association. This effect coincides with those reported in Vero cells (El Golli-Bennour *et al.*, 2010) and it suggests that either the mycotoxins do not share cell pathways in their toxic action, or if they share some steps, it is expected that they would at least have additive effects (Speijers, 2004). With respect to the genotoxicity, the combined treatments showed a significant decrease in DNA damage, not only in breaks and AP sites but also in FPG-sensitive sites, when compared to the AFB1 treatment. On the other hand, ROS increased when mycotoxins coexist inside cells. A possible hypothesis may be that AFB1 and OTA compete for the same CYP enzymes that represent a bioactivation route for AFB1. If OTA had much more affinity for the CYPs involved, it would displace AFB1, forcing the creation of secondary routes of metabolism such as ROS induction. There would be less AFB1 bioactivated molecules (AFB1-epoxide) for attacking and damaging DNA, and as a result, the combination of these two toxins would yield less direct DNA damage.

In vitro protection against oxidative damage

Natural constituents such as polyphenols have been widely studied as protectors against cytotoxicity and oxidative damage caused by exposure to harmful substances. With respect to mycotoxins, polyphenol protection evidence is not conclusive. Muto *et al.* (2001) found that epicatechin (EC) partially inhibited the bioactivation of AFB1 in CYPs expressing bacteria. However, Lee *et al.* (2005) could not find any inhibition in the epoxide synthesis when AFB1 was mixed with mouse liver microsomal proteins and catechins. Costa *et al.* (2007) found excellent protective effects of catechin derivatives against OTA cytotoxicity, acting as good ROS scavengers in a pig kidney cell line (LLC-PK1). However, Hundhausen *et al.* (2005) found no protection from OTA induced cytotoxicity in Hep G2 cells.

A polyphenol-enriched extract (PECE) was obtained from cocoa seeds. The extract contained the highest proportion of epicatechin procyanidins compared to other phenolic extracts from plant sources (Touriño *et al.*, 2005; Jerez *et al.*, 2007; Touriño *et al.*, 2008; Touriño *et al.*, 2011), and it showed effective antiradical activity in cell-free systems. These antioxidant properties encouraged us to test the extract in a biological system (Hep G2 cell line). The PECE, like other extracts with non-galloylated procyanidin oligomers (Ugartondo *et al.*, 2007), was a weak cytotoxic mixture (at the limit of solubility, cell survival was higher than 55%) and induced ROS only at cytotoxic concentrations (cell survival less than 80%). Therefore, it was selected as a promising antioxidant candidate for reducing the damage produced by AFB1 and OTA.

With regard to AFB1, the PECE did not reduce the cytotoxicity. Moreover, the PECE was not very effective on ROS scavenging and only reduced the ROS levels at the highest concentration with 50 mg/L. This may indicate that radical formation is not the principal mechanism of AFB1 cytotoxicity, although it does play a role as a secondary route of action (Bedard and Massey, 2006). With regard to OTA treatments, both in isolated and combined treatments with AFB1, cell survival improved. Coinciding with this, PECE was very effective reducing ROS formation in all of the OTA and combined treatments, showing that OTA ROS induction is directly related with its cytotoxicity (Arbillaga *et al.*, 2007a; Arbillaga *et al.*, 2007b). Surprisingly, the greatest reduction of ROS was observed with the "prooxidant" concentration of 50 mg/L. This observation highlights the dual pro-oxidant/antioxidant activity of the molecules with multiple phenol units according to the redox properties of the molecules that interact with them. Therefore, polyphenols extracted from cocoa

have a good antioxidant activity and may be efficient in reducing the generation of ROS produced by mycotoxins or other oxidant agents.

In vivo toxicity: toxicokinetic approach and combined genotoxicity

In order to give a global response regarding the genotoxicity *in vivo* of AFB1 or OTA alone and combined, it is essential to answer more questions than just in the cases where there is DNA damage after the treatments. It is important to describe the general status of the animals. In addition, the serum biochemical parameters may give clues regarding how organs have been affected and the histopathological status of target organs may show the kind of cellular damage that has occurred during molecule metabolism. The ICH guideline S3A highlights the need to integrate toxicokinetics into toxicity testing, which should aid in the interpretation of the toxicology findings (ICH, 1994). Basic toxicokinetic parameters provide information on the potential for accumulation of the test substance in tissues and/or organs and the potential for induction of biotransformation (OECD, 2009). Moreover, in our opinion, the DNA damage should be studied not only in bone marrow or lymphocytes but also in expected target tissues.

In order to determine the genotoxicity of AFB1, OTA, and the combination of both, it was decided to first study the toxicity and the toxicokinetic behavior of the mixture, because there was no previous data available regarding the possible interactions during absorption, metabolism or toxic effects. It is essential to use well defined and fully validated analytical methods in order to obtain reliable results that can be satisfactorily interpreted (Bressolle *et al.*, 1996).

Validation of a method for quantifying AFB1 and OTA in plasma and tissues

A rapid and simple method for the simultaneous quantification of AFB1 and OTA in rat plasma, liver and kidney by UHPLC-FLD has been successfully set up and validated. The extraction method, calibration curves and chromatographic conditions were common for the three matrices.

The sampling has been inspired from the work described by Vettorazzi *et al.* (2008) for detecting OTA in plasma, liver and kidney of rats. Once the samples were collected, the mycotoxins were extracted satisfactorily with a mixture of ACN:formic acid (99:1) in a ratio 1:3 (1 part of sample: 3 parts of extracting mixture). The simultaneous extraction has been achieved only starting from 100 µL of plasma or 25 mg of tissue and covering a range for AFB1 and OTA equivalent to 2-6,000 µg/L in rat plasma and 8-2,400 µg/kg in rat kidney and liver. Only one solvent step extraction

was needed, avoiding immunoaffinity column clean up and toxic solvents such as chloroform, ethyl acetate or dichloromethane which cause health and environmental hazards.

Chromatographic conditions were investigated in order to achieve the best separation and resolution of peaks so as to allow quantification. This method used a low-volume column that permits working under high pressure conditions, thereby giving high resolution in short time assays. It started with an isocratic set which permits the elution of the polar components extracted with the mycotoxins. Due to its polar properties, AFB1 appeared early in the chromatogram, whereas OTA needed an increase in the organic proportion of the eluent. Both mycotoxins have fluorescent properties; however, AFB1 suffers quenching in aqueous solvents; therefore, derivatization reactions were necessary for quantifying low levels (Kok, 1994; Ibáñez-Vea *et al.*, 2012). The derivatization was performed with a photo-chemical reactor made with a knitted coil and a mercury lamp placed in line just after the column (Joshua, 1993). The PHRED makes derivatization fast and easy, and minimizes intervention of the analyst. The AFB1 signal was approximately 15 times more intense with the lamp on while OTA response remained unaffected.

Once the method was set up, it was validated according to the criteria suggested by ICH Q2 (R1) (2005) and the AEFI (2001): selectivity, stability, linearity, precision, accuracy, recovery, robustness and limits of quantification and detection.

The method was selective for kidney and liver because no interference peaks appeared at the retention time of AFB1 or OTA in blank samples. In the case of plasma, no interference peaks appeared at the retention time of AFB1; however, basal OTA levels were detected in blank plasma due to natural contamination of the feed (Mantle, 2008).

With regard to stability, the working solutions remained stable up to 4 weeks at -20ºC, and spiked samples stored at -80ºC were stable during 6 months as observed by Vettorazzi *et al.* (2008). Finally, prepared extracts, ready to inject in the HPLC, were stable for 15 h in the tray at 4ºC without light.

Linearity has been assessed in a wide range of concentrations in order to include high and low levels so as to characterize the entire kinetic curves. The four calibration curves that were generated showed a good linear relationship between response (area of the peaks) and the respective AFB1 or OTA concentrations. All of the requirements for linearity have been met for the two concentration

intervals of each mycotoxin. Precision (RSD) and accuracy (SE) of the linearity showed adequate values, less than 10% in the whole interval (at the LOQ level, less than 15%).

The recovery for each matrix was very efficient, between 93 - 96% for AFB1 and between 94-96% for OTA. Moreover, the RSDs obtained in the within-day and between-day experiments were below 10% in each case (at the LOQ level, below 15%), thereby demonstrating the precision of the analytical procedure. The recovery obtained in this method for AFB1 was successful for every matrix and higher than in other HPLC methods found in the reference literature (Gregory, 1982; Lamplugh, 1983a; Lamplugh, 1983b). In terms of OTA, the recovery was consistent with other methods used for its analysis in plasma or serum (Vettorazzi *et al.*, 2008; Zepnik *et al.*, 2003), or higher in liver and kidney (Vettorazzi *et al.*, 2008; Zepnik *et al.*, 2003; De Saeger *et al.*, 2004).

For both mycotoxins, the LOQs were 2 μg/L in plasma and 8 μg/kg in liver and kidney; they were similar to or better than others found in the reference literature for HPLC methods or radioactivity detection which used 2 to 100 times more sample volume (Vettorazzi *et al.*, 2008; Gregory, 1982; Lamplugh, 1983a; Lamplugh, 1983b; De Saeger *et al.*, 2004; Valenta, 1998; Coulombe and Sharma, 1985). The calculated LODs for AFB1 were as follows: 0.1 μg/L in plasma and 0.01 μg/kg in kidney and liver; the calculated LODs for OTA were: 0.3 μg/L in plasma and 0.01 μg/kg in kidney and liver.

In the study of robustness, changes in the column batch, the pressure or the column temperature modified the retention times of the peaks but did not affect the quantification. However, an increase in the pH yielded a decrease in the OTA signal, whereas AFB1 signal remained stable. OTA has been described as a weak acid and it is important to maintain a pH below 4.4 in order to assure that the OTA molecule is in its protonated form (Valenta, 1998). Moreover, it is very important to keep samples in the dark once when they are obtained or being manipulated due to the fact that AFB1 degrades easily when exposed to light.

The method was successfully applied to real samples obtained from rats that received a mixture of AFB1 and OTA (0.5 mg/kg bw of AFB1 and 0.1 mg/kg bw of OTA) in a single dose by oral gavage. Therefore, the analytical method was suitable for the simultaneous detection and quantification of both mycotoxins during toxicokinetic and toxicological studies.

Toxicokinetic approach of the combination of mycotoxins

There are no previous *in vivo* studies regarding the possible interactions between AFB1 and OTA regarding their absorption, metabolism or elimination. This study intended to obtain data regarding the toxicity of AFB1 and OTA when they are administered together, to give some basic kinetic parameters, and to determine if either of the toxins interacts in the kinetics of the other. Fisher 344 rats were administered a mixture 0.25 mg/kg bw of AFB1 and 0.5 mg/kg bw of OTA which corresponds approximately to the 3% of the LD_{50} of each mycotoxin alone. Samples were collected from 10 min up to 96 h.

Although there were no signs of general toxicity during the experiment, the biochemical and the histopathological results pointed to acute toxicity in liver. However, the liver started a repairing process after 72 h. Its effects were evident because the transaminases (ALT, AST), which rose significantly in 24 h, almost returned to normal values after 96 h. Moreover, at that time, the regenerative phenomena were evident by the numerous binucleated cells that were present. These findings were consistent with the observations made by Rati *et al.* (1981) regarding acute toxicity of AFB1, and it appeared that the AFB1 and/or its toxic metabolites were eliminated (metabolized and excreted) in a range of 48 - 96 h. No remarkable toxicity was observed in kidneys or immunological organs (spleen, thymus and Peyer's patches) so mycotoxins did not cause acute toxicity at this dose, as expected. In conclusion, the acute toxic effects observed after the combined treatment of AFB1 and OTA were mostly due to AFB1.

With regard to the evident signs of toxicity in liver, high AFB1 levels might have been expected in plasma and liver at least at the collected early time points and detectable levels in plasma during at least 72 h. Previous studies of AFB1 kinetics in rats described a two-compartment model with long plasma half lives higher than 50 hours after quantifying AFB1 alone or mixed with its metabolites (Coulombe and Sharma, 1985; Firmin *et al.*, 2010). However, the absorption of AFB1 was very fast and it was rapidly metabolized. Only an astonishing 1% of the dose was found in plasma after 10 min and 0.4% was found after 30 min, with the liver levels lower than the LOQ (8 μg/kg) (Corcuera *et al.*, 2011b) at all times, and AFB1 was undetectable in kidney. Moreover, almost none of the un-metabolized AFB1 was distributed in plasma outside the liver to the kidneys. In the plasma chromatograms, new peaks appeared in the front that could be related with AFB1 metabolites because they have not been detected in recent OTA kinetic studies (Vettorazzi *et al.*, 2007; Vettorazzi *et al.*, 2009; Vettorazzi *et al.*, 2011) or during the validation of the method with

spiked plasma samples. The new peaks had a maximum at 30 min, decreased in a time-dependent way until 24 h, and then were undetectable. Unfortunately, the front peaks of liver and kidney samples were too big to be able to find little increases of the metabolites. Our observations described an extremely fast uptake and metabolism of AFB1 which is completely metabolized in 30 min and eliminated in 24 h. In humans, Jubert *et al.* (2009) observed a rapid uptake (T_{max} = 1 h), and urinary elimination of 95% of the dose in 24 h, which is more consistent with our findings; in this work, they were not able to differentiate between AFB1 and its metabolites.

The OTA concentration profiles in plasma, liver and kidney and some of the kinetic parameters are similar to the ones that Vettorazzi *et al.* (2010) obtained under the same conditions of this experiment but only with OTA. The maximum concentration in plasma was obtained after 2 h and corresponded to 87% of the initial dose that was similar to the 83% that Vettorazi *et al.* (2010) reported. However, an apparent delay in the elimination of OTA resulted in a higher plasma half-life. This event might be explained because the liver had suffered acute damage which had affected its functions in the metabolism of OTA, and the OTA elimination occurs mainly in the liver compartment. In this case, higher doses of OTA (when compared to OTA alone) would have been expected in plasma, liver and kidney. However, no significant differences were observed in tissues after 24 h between OTA alone and OTA administered with AFB1. Upon comparing the OTA profile obtained with other experiments and models of OTA kinetics, the differences in the OTA elimination could be related more to the age and the sex of the rats than to the presence of AFB1 in the system (Vettorazzi *et al.*, 2007; Vettorazzi *et al.*, 2010; Vettorazzi *et al.*, 2011).

Combined genotoxicity in vivo

The ICH S2 (R1) draft guideline on genotoxicity testing and data interpretation recommends integrating *in vivo* genotoxicity assays into preclinical toxicity studies when the dose levels are justifiable and the protocols are compatible (ICH, 2011). Moreover, if several *in vivo* genotoxicity tests could be integrated into one single study; this would reduce animal use. With this aim, the *in vivo* micronucleus assay in bone marrow and the *in vivo* comet assay in liver and kidney have been performed. Going a step forward, in this study, general toxicity and organ exposure data were taken into account in order to give a wider perspective of the results.

In the previous study, the toxicokinetic profile of a single oral dose of AFB1+OTA was studied. It showed that the absorption of AFB1 was extremely fast, the metabolism occurred

exclusively in liver, and its metabolites were excreted in 24 h. With regard to OTA, this mycotoxin was also rapidly absorbed, followed by a long elimination process with high levels in plasma and tissues during more than 96 h. General liver deterioration was observed but there was no toxicity in kidney or any other organs. All these signs suggested mild acute toxicity due to AFB1. This valuable information helped us to design the dose range and the time endpoints of the *in vivo* genotoxicity tests.

Although Bowen *et al.* (2011) suggested a design for the comet and MN assays giving three repeated administrations (0, 24, 45 h), in this study the mycotoxins, alone or combined, were administered only once, because we tried to avoid excessive acute toxicity in the liver. Excessive toxicity can confound the ability to determine genotoxicity in the comet assay (Vasquez, 2010). The OECD guideline suggests using both sexes (OECD, 1997b), but this study was carried out using only male rats due to the following reasons: Madle *et al.* (2005) observed that AFB1 induced more MN in male rats and mice than in females and OTA is more carcinogenic in male rodents (Castegnaro *et al.*, 1998; EFSA, 2006). One oral dose of AFB1 (0.25 mg/kg bw), OTA (0.5 mg/kg bw) or the mixture was administered to F344 rats. After 3 h or 24 h, samples were collected in order to perform the comet assay in liver and kidney and the micronucleus test in bone marrow, and so as to check the biochemical status in plasma and to study the histopathological changes in liver and kidney. In addition, samples of liver and kidney were saved in order to perform microarray analysis on the gene expression changes occurring during the treatments, but this study is still in progress and it is not a part of this thesis.

No signs of general toxicity were observed but the biochemical analysis revealed mild acute liver damage after 24 h in the groups treated with AFB1 as expected. Surprisingly, the biochemical liver alterations were more pronounced when AFB1 was administered alone than when it was co-administered with OTA. The histopathological findings revealed necrosis and inflammation in the livers of rats treated with AFB1 alone or combined, and once more, the liver damage was less pronounced in the combined treated samples. A decrease in the absorption or distribution of mycotoxins was discarded because plasma and tissue levels were similar at 3 h and 24 h in groups treated with one or both mycotoxins and consistent with the toxicokinetic study and other published data (Vettorazzi *et al.*, 2009; Vettorazzi *et al.*, 2011). Therefore, the reduced toxic effects might be due to changes in the bioactivation and/or metabolism of AFB1 as was suggested in the case of *in vitro* experiments using human liver cells (Hep G2) (Corcuera *et al.*, 2011a).

The genotoxic capacity of AFB1 is already known and the micronucleus test revealed that AFB1 was cytotoxic in the bone marrow after 3 h and genotoxic after 24 h. OTA did not induce micronuclei, but one single dose was sufficient for causing toxicity in bone marrow after 3 h. Therefore, it was confirmed that mycotoxins reached the bone marrow and that the doses were high enough so as to provoke an effect as the OECD guideline recommends (OECD, 1997b). The AFB1+OTA treatment was positive but the cytotoxicity and number of micronuclei in the bone marrow were less than in the treatment of AFB1 alone.

With regard to the comet assay carried out on the target organs, no significant direct DNA breaks were observed in liver or kidney, most likely due to the cell repairing processes, but very interesting results were obtained with the restriction enzymes (endo III and FPG).

In the livers of AFB1 treated groups, a significant increase in FPG-SS was observed after 3 h, and the injuries persisted during 24 h but with less intensity; in addition, endoIII-SS appeared at this time. The FPG-SS may correspond to the imidazole AFB1 formamidopyrimidine opened rings (AFB1-FAPY) after the attack of the bioactivated AFB1 on DNA (Bedard and Massey, 2006; Corcuera *et al.*, 2011a). Moreover, FPG enzyme also detects 8-hydroxydeoxyguanosine (8-oxodG) (Collins and Dusinska, 2002) so it is possible that AFB1 induced oxidative damage as Shen *et al.* (1995) had observed in rats. It is difficult to establish the degree of the contribution of the oxidative stress to the observed genotoxicity, but both pathways contribute to the DNA damage (Corcuera *et al.*, 2011a). OTA did not cause effects on livers. We expected that OTA, which is a GSH depletor, among other enzymes involved in free-radical scavenging *in vivo* (O'Brien and Dietrich, 2005), enhanced AFB1 genotoxicity *in vivo*, because AFB1-epoxide is converted into AFB1-GSH by the cellular glutathione-S-transferase and the level of this enzyme is critical for modulating AFB1 toxicity (Raj *et al.*, 1998). On the contrary, the genotoxic damage in liver with the mixture was less than AFB1 alone after 3 h. The hypothesis that OTA hinders the AFB1 bioactivation, reducing the direct DNA damage as in the case of *in vitro,* gains more strength (Corcuera *et al.*, 2011a).

With regard to the kidney results, OTA induced FPG-SS when administered alone or in combination with AFB1. This increase is consistent with the works of Zeliezic *et al.* (2006) and Domijan *et al.* (2006) which supported the idea that oxidative stress is likely to be responsible for the DNA damage, and with the results of Mally *et al*, in 2005 and 2006, which suggested that the increase in FPG-SS and the absence of DNA adduct pointed to a non-direct reactive DNA mechanism of toxicity. The oxidative DNA damage detected was maintained after 24 h. OTA has long half live in

plasma and high apparent volume of distribution and these two facts may account for prolonged exposure of cells to the oxidative effects of OTA. No DNA damage was observed in the kidneys of the AFB1 treated groups, and OTA oxidative damage in kidney was not modified by the presence of AFB1, so we could say that the oxidative damage observed in kidneys was mostly due to OTA. OTA did not induce MN in bone marrow although it was cytotoxic in that organ and it induced DNA oxidative damage in kidney, its target organ.

In conclusion, AFB1 was positive for the bone marrow MN assay and OTA was negative. Co-exposure reduced both general toxicity and genotoxicity. In target organs, AFB1 increased FPG-SS in liver and OTA had a similar effect in kidney. Co-exposure decreased AFB1 effect on liver but had no effect on kidney. Therefore, it has been demonstrated that simultaneous exposure to both mycotoxins modifies toxic effects. Moreover, the combination of classical genotoxicity tests such as the MN assay and new techniques such as the comet assay, which can be applied to tissues other than bone marrow or blood, gives complementary results. This contributes to the 3Rs strategy by increasing the information that can be obtained from a single animal experiment, and also improves the risk assessment process by producing results that can be interpreted in a more rational way.

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Capítulo 9/Chapter 9

Conclusiones /Conclusions

CONCLUSIONES

1. Con respecto a la genotoxicidad *in vitro* en células humanas Hep G2:

   1.1. La aflatoxina B1 fue mas citotóxica que la ocratoxina A y el efecto conjunto de ambas fue aditivo.

   1.2. La aflatoxina B1 fue genotóxica dependiente de activación metabólica. Se detectaron roturas en el ADN y lugares sensibles a la FPG. Sin embargo, la ocratoxina A resultó no genotóxica en las mismas condiciones.

   1.3. La coexposición a una mezcla de aflatoxina B1 y ocratoxina A disminuyó el efecto genotóxico de la aflatoxina B1, tanto en las roturas directas como en el daño oxidativo.

   1.4. Ambas micotoxinas fueron capaces de inducir especies reactivas de oxígeno intracelulares. El tratamiento conjunto indujo más especies que los tratamientos individuales, observándose una asociación aditiva.

   1.5. El extracto de cacao enriquecido en polifenoles no fue efectivo frente a la aflatoxina B1, pero redujo significativamente la cantidad de especies reactivas de oxígeno en las células tratadas con ocratoxina A o la mezcla de ambas micotoxinas. Esto sugiere que los polifenoles del cacao son buenos candidatos como agentes antioxidantes.

2. Con respecto a la toxicidad y el comportamiento cinético de las micotoxinas en rata:

   2.1. Se ha desarrollado y se ha validado un método rápido y sencillo para la determinación de aflatoxina B1 y ocratoxina A simultáneamente en plasma, hígado y riñón. La validación del método se ha realizado conforme a los siguientes criterios: selectividad, linealidad, precisión y exactitud, límites de detección y cuantificación, recuperación, estabilidad y robustez. En todos los casos se han obtenido resultados aceptables según los objetivos establecidos.

   2.2. La pequeña cantidad de muestra necesaria, el bajo volumen de disolventes utilizado, así como los límites de detección y cuantificación alcanzados hacen que este método haya podido ser empleado en estudios cinéticos en rata.

2.3. La mezcla 0,25 mg/kg pc de aflatoxina B1 y 0,5 mg/kg pc de ocratoxina A provocó un cuadro de hepatotoxicidad aguda que se recuperó al cabo de 96 h. Se puso de manifiesto por un aumento de transaminasas y por el estudio histológico.

2.4. El estudio cinético demostró que la absorción y metabolismo de la molécula de aflatoxina B1 son extremadamente rápidos, ya que sólo se observo un 1% de la dosis en plasma tras 10 min, niveles inferiores al 10% de la dosis en hígado tras 8 h, fue indetectable en riñón y los metabolitos desaparecieron del organismo en 24 h.

2.5. Parece que la presencia de aflatoxina B1 no afecta a la cinética de la ocratoxina A ya que el valor máximo y el perfil cinético observado fueron comparables a los encontrados en estudios cinéticos previos de la ocratoxina A en las mismas condiciones.

3. Con respecto a la genotoxicidad conjunta *in vivo*:

3.1. La presencia de ocratoxina A disminuyó la hepatotoxicidad aguda de la aflatoxina B1 que se puso de manifiesto por un descenso de las transaminasas, menor daño histológico y menores pesos relativos del hígado.

3.2. La AFB1 indujo muy significativamente micronúcleos y toxicidad en médula osea. Sin embargo, la ocratoxina A fue tóxica en médula osea pero no fue capaz de inducir micronúcleos a esa dosis.

3.3. La presencia de ocratoxina A redujo la citoxicidad y el porcentaje de micronúcleos inducido por la aflatoxina B1.

3.4. El ensayo del cometa permitió detectar una genotoxicidad órgano específica de tal manera que la aflatoxina B1 indujo significativamente lugares sensibles a la FPG en hígado y la ocratoxina A en riñón.

3.5. Las lesiones detectadas por la enzima en el hígado corresponden en su mayoría y probablemente al aducto AFB1-formamidopirimidina, fruto de un ataque directo del epóxido al ADN. Sin embargo, las lesiones detectadas en riñón fueron probablemente 8-oxoguaninas debidas al daño oxidativo inducido por la OTA.

3.6. La presencia de ocratoxina A disminuyó las lesiones en el ADN provocadas por la aflatoxina B1 en el hígado, aunque la coexposición no tuvo efectos en el riñón.

4. En general, la exposición a ambas toxinas simultáneamente supuso una disminución de la genotoxicidad provocada por la aflatoxina B1 tanto en modelos *in vitro* como *in vivo*.

CONCLUSIONS

1. With regard to genotoxicity *in vitro* in Hep G2 human cell line:

   1.1. Aflatoxin B1 was more cytotoxic than ochratoxin A and the combined effect was additive.

   1.2. Aflatoxin B1 was genotoxic with metabolic activation. DNA strand breaks and FPG sensitive sites were detected. However, ochratoxin A was not genotoxic under the same conditions.

   1.3. Co-exposure to a mixture of aflatoxin B1 and ochratoxin A reduced the genotoxicity of aflatoxin B1, in the direct strand breaks as well as in the oxidative damage.

   1.4. Both mycotoxins were able to induce intracellular reactive oxygen species. The combined treatment induced more reactive species than the individual treatments, observing additive association.

   1.5. The polyphenol-enriched cocoa extract was not effective against aflatoxin B1 but it significantly reduced the reactive oxygen species induced by ochratoxin A or the mixture of mycotoxins. These observations suggest that cocoa polyphenols would be good candidates as antioxidant agents.

2. With regard to the toxicity and toxicokinetic profile of both mycotoxins in rats:

   2.1. A fast and simple analytical method has been developed and validated for the simultaneous determination and quantification of aflatoxin B1 and ochratoxin A in rat plasma, liver and kidney. The validation of the method has been performed based on the following criteria: selectivity, linearity, precision and accuracy, limits of detection and quantification, recovery, stability and robustness. In accordance with the validation objectives established, acceptable results have been obtained in each case.

   2.2. The small amount of sample needed, the low volume of solvent used, and the limits of detection and quantification achieved made this method useful for kinetic studies in rats.

2.3. The mixture of 0.25 mg/kg bw of aflatoxin and 0.5 mg/kg bw of ochratoxin A caused acute hepatotoxicity in treated rats which recovered after 96 h. This recovery was revealed by an increase in transaminases and by the histology observations.

2.4. The kinetic study showed that the absorption and metabolism of aflatoxin molecule are extremely fast, since only 1% of the initial dose was observed after 10 min and less than 10% of the dose was observed in liver after 8 h. It was undetectable in kidney and metabolites disappeared from the organism within 24 hours.

2.5. It appears that the presence of aflatoxin B1 does not affect the kinetics of ochratoxin A because the maximum value and the kinetic profile observed were similar to those found in previous kinetic studies conducted on ochratoxin A under the same conditions.

3. With respect to the combined genotoxicity *in vivo*:

3.1. The presence of ochratoxin A decreased the acute hepatotoxicity of aflatoxin B1 which was demonstrated by a decrease of transaminases, a reduction in histological damage and lower relative weights of the liver.

3.2. Aflatoxin B1 induced micronuclei and cytotoxicity in bone marrow very significantly.

3.3. The presence of ochratoxin A reduced cytotoxicity and the percentage of micronuclei induced by aflatoxin B1 in bone marrow.

3.4. The comet assay made the detection of specific organ genotoxicity possible so that aflatoxin B1 significantly induced FPG-sensitive sites in liver and ochratoxin A in kidney.

3.5. The majority of lesions detected by the enzyme in the liver are probably the adduct AFB1-formamidopyrimidine as a result of a direct attack of the epoxide on DNA. However, kidney lesions were 8-oxoguanines probably due to oxidative damage induced by OTA.

3.6. The presence of ochratoxin A decreased DNA damage induced by aflatoxin B1 in the liver, although the co-exposure had no effect on the kidney.

4. In general, the exposure to both toxins simultaneously resulted in a decrease of the genotoxicity of aflatoxin B1 in both *in vitro* and *in vivo* models.