VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

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

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Efectos tóxicos de la zearalenona y sus metabolitos en líneas celulares

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

CERTIFICAN QUE:

Dª Elena Tatay Dualde, licenciada en Farmacia, ha realizado bajo nuestra dirección el trabajo que lleva por título: "*Efectos tóxicos de la zearalenona y sus metabolitos en líneas celulares*". Y autorizamos la presentación para optar al título de Doctor.

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

Burjassot (Valencia), 2017

Dra. Guillermina Font Pérez

Dra. María José Ruiz Leal

La presente tesis doctoral ha dado lugar a 5 artículos, publicados o que se publicaran en las siguientes revistas:

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2. Elena Tatay, Giuseppe Meca, Guillermina Font, Maria-Jose Ruiz. Cytotoxic and interactive effects of zearalenone, α-zearalenol and β-zearalenol and formation of metabolites in HepG2 cells. Revista de Toxicología, 31 (2015) 187-195.

3. Elena Tatay, Guillermina Font, Maria-Jose Ruiz. Cytotoxic effects of zearalenone and its metabolites and antioxidant defense in CHO-K1 cells. Food and Chemical Toxicology, 96 (2016) 43-49. Impact factor: 3.584

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La presente Tesis Doctoral se engloba dentro de los siguientes proyectos y red:

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

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

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

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ABREVIATURAS

AEMPS: Agencia Española del Medicamento y Productos Sanitarios
AFB1: Aflatoxina B1
AFM1: Aflatoxina M1
AFs: Aflatoxinas
ALP: Ensayo de la fosfatasa alcalina
AR: Receptor androgénico humano
BDB: Dominio central de unión al ADN
BEA: Beauvericina
BRL3A: Células de hígado de rata
BrdU: 5-bromo-2´-deoxiuridina
Caco-2: Células de adenocarcinoma de colon humano
CAT: Catalasa
CCL3: Células de carcinoma del cuello uterino
CI: Índice de combinación
CIT: Citrinina
CHO-K1: Células de ovario del hámster chino
CL: Ensayo de la lucigenina
CYP450: Citocromo p450
C5-O: Células de queratinocitos de ratón
DBD: Dominio central de unión al ADN
DCF: 2,7-diclorofluoresceína
DCFH: 2,7-diclorodihidrofluoresceina
DMEM: Mezcla de nutrientes para las células (Dulbecco's modified Eagle médium)
DMSO: Dimetil sulfoxido
DON: Deoxinivalenol

ECHA: Agencia Europea de Productos Químicos

ECVAM: Centro Europeo para la Validación de Métodos Alternativos

EC_{50}: Concentración efectiva media

EDC: Compuestos disruptores endocrinos

EFSA: Autoridad Europea de Seguridad Alimentaria

EMA: Agencia Europea de Medicamentos

ENs: Eniatinas

E2: 17β-estradiol

FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura

FB1: Fumonisina B1

FB2: Fumonisina B2

FDA: Fluoresceína diacetato

GPx: Glutatión peroxidasa

GR: Glutatión reductasa

GSH: Glutatión

GSSH: Glutatión oxidado

HAM-F12: F-12 mezcla de nutrientes para las células

HCT116: Células de carcinoma de colon

H_2-DCFDA: 2',7'-diclorodihidrofluoresceína diacetato

HEK293: Células embrionarias de riñón

HeLa: Células de carcinoma de cuello de útero humano

HEPES: Tampón fosfato

HepG2: Células del hepatoma humano

H_2O_2: Peróxido de hidrogeno

HT-2: Toxina HT-2

H295R: Células de carcinoma adrenocortical humano

H9c2: Células cardiacas

IARC: Agencia Internacional de Investigación sobre el cáncer

IPCS: Programa Internacional de Seguridad Química, Pesticidas y Cosméticos

Jurkat T: Células de linfoblastoides humanas

LBD: Dominio de unión C-terminal

LC-MS: Espectrometría de masas

LC/MS/LIT: Cromatografía líquida acoplada a espectrometría de masas con trampa de iones lineal

LD: Límite de detección

LHD: Lactato deshidrogenasa

LMA: Agarosa de bajo punto de fusión

LQ: Límite de cuantificación

MA-10: Células tumorales de ratón

MCF-7: Células de cáncer de mama

MLTC-1: Células tumorales de ratón

MON: Moniliformina

MTT: Bromuro de 3[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio

$NaHCO_3$: Bicarbonato sódico

Na_2-EDTA: Ácido etilendiaminotetraacético sódico

NAT: Nitroazul de tretazolium

NE: No especificado

NEAA: Aminoácidos no esenciales

NIV: Nivalenol

NTD: Dominio N-terminal

$O^{-\bullet}_2$: Radical anión superóxido

•OH: Radical hidroxilo

OCED: Organización para la Cooperación Económica y el desarrollo

OPT: o-ftaldehído

OT: Ocratoxina

OTA: Ocratoxina A

PAT: Patulina

PBS: Tampón fosfato salino

pc: Peso corporal

PC: Proliferación celular

PCR: Reacción en cadena de la polimerasa

PE: Efecto proliferativo

PER: Potencial estrogénico relativo

PI: Índice proliferativo

PK15: Células de epitelio de riñón de cerdo

PMNs: Neutrófilos

RAW 264.7: Macrófagos

RE: Receptor estrogénico

REs: Receptores estrogénicos

REα: Receptor estrogénico α

REβ: Receptor estrogénico β

RGA: Ensayo gen reportero de la luciferasa

RLU: Unidades relativas de luz

RN: Rojo neutro

RPE: Efecto proliferativo relativo

ROS: Especies reactivas de oxigeno

RSDr: Desviación relativa estándar

SFB: Suero fetal bobino

SHGB: Globulina de unión de hormonas sexuales

SH-SY5Y: Células de neuroblastoma humano

SOD: Superóxido dismutasa

SRB: Ensayo de la sulforhonamina B

TCA: Ácido tricloracético

T-2: Toxina T-2

T47D: Células de carcinoma humano de mama

UE: Unión Europea

UGT: Enzima uridindifosfato-glucorinil transferasa

Vero: Células de riñón de mono

V79: Células de fibroblastos de pulmón de hámster chino

WST: Tetrazolium soluble en agua

ZEA: Zearalenona

α-ZAL: α-Zearalanol

β-ZAL: β-Zearalanol

α-ZOL: α-Zearalenol

β-ZOL: β-Zearalenol

3Rs: Principios de las tres R

RESUMEN

La zearalenona (ZEA) y sus metabolitos α-zearalenol (α-ZOL) y β-zearalenol (β-ZOL), son metabolitos secundarios de hongos del genero *Fusarium*. La ZEA se absorbe y se metaboliza a α-ZOL y β-ZOL. Tanto la ZEA como sus metabolitos producen sus efectos tóxicos por la interacción con los receptores endocrinos aunque no es el único mecanismo por el cual pueden producir toxicidad. Se sabe que, además de disruptores endocrinos la ZEA y sus metabolitos son inmunotóxicos, hepatotóxicos y hematotóxicos causando alteraciones en el sistema reproductivo.

La citotoxicidad de la ZEA y sus metabolitos ha sido determinada por distintos tiempos de exposición en diferentes células de mamíferos obteniéndose diferentes valores de IC_{50}.

Se investigó la conversión de ZEA y sus metabolitos en estos u otros derivados de ZEA por células CHO-K1 y HepG2 observando diferentes derivados de la ZEA y sus metabolitos. La exposición a estas micotoxinas causa estrés oxidativo observándose un aumento de las especies reactivas de oxigeno (ROS) que es proporcional al tiempo de exposición y la concentración de las micotoxinas. Se ha visto que este aumento de ROS conduce a una alteración de las vías metabólicas, disminución de los antioxidantes celulares y provoca que se oxiden estructuras macromoleculares, entre las que se encuentran las proteínas y lípidos de las membranas celulares y el ADN.

Se evaluó la actividad estrogénica de la ZEA y sus metabolitos usando células de cáncer de mama humano (MCF-7) y se observó un aumento de esta actividad siendo el α-ZOL el que producía un mayor aumento de la actividad estrogénica.

Se determinó si la ZEA y sus metabolitos activan los mecanismos de defensas antioxidantes en las células CHO-K1 y HepG2. Para ello, se determinó la actividad del glutatión (GSH) y de las enzimas catalasa (CAT), superóxido dismutasa (SOD) y glutatión peroxidasa (GPx) tras exponer las células durante 24 h en las células CHO-K1 y HepG2. Los resultados mostraron una disminución de los niveles de GSH y de la actividad de la catalasa. Mientras que se evidenció un aumento en la actividad de la SOD y GPx. Todos estos efectos tóxicos pueden conducir a un riesgo en la salud humana.

ABSTRACT

Zearalenone (ZEA) and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) are secondary metabolites of *Fusarium* fungy. ZEA is absorbed and metabolized to α-ZOL and β-ZOL. Boths ZEA and its metabolites produce their toxic effects through interactions with endocrine receptors, although it is not the only mechanism by which they can produce toxicity. It is known that in addition to endocrine disruptors ZEA and its metabolites are immunotoxic, hepatotoxic and hematotoxic causing alterations in the reproductive system.

The cytotoxicity of ZEA and its metabolites has been determined by different exposure times in different mammalian cells obtaining different IC_{50} values.

It was investigated the conversion of ZEA and its metabolites into these or other ZEA derivatives in CHO-K1 and HepG2 cells by observing different derivates of ZEA and its metabolites. The exposure to these mycotoxins causes oxidative stress observing an increase of the reactive oxygen species (ROS) that is proportional to the time of exposure and the concentration of the mycotoxins. It is seen that this growth of ROS leads to an alteration of metabolic pathways, a decrease in cellular antioxidants and causes oxidation of macromolecular structures, among which are the proteins and lipids of cell membranes and DNA.

The estrogenic activity of ZEA and its metabolites was evaluated using human breast cancer cells (MCF-7) and an increase of this activity was observed. α-ZOL was the one that produced a greater increase of the estrogenic activity.

It was determined if ZEA and its metabolites activate the mechanisms of antioxidant defenses in CHO-K1 and HepG2 cells. For this purpose, the

activity of glutathione (GSH) and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined after exposed the cells for 24 h in CHO-K1 and HepG2 cells. The results showed a decrease in GSH levels and a decrease in catalase activity. While an increase in the activity of SOD and GPx was evidenced. All these toxic effects can lead to a risk to human health.

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. GENERALIDADES DE LAS MICOTOXINAS

Las micotoxinas son metabolitos secundarios producidos por hongos filamentosos. Los principales hongos productores de micotoxinas son de los géneros *Aspergillus, Fusarium, Penicillium y Claviceps*. Los hongos producen las micotoxinas como sistema de defensa contra los insectos, microorganismos, nematodos y otros organismos (Marin y col. 2013).

En la Tabla 1 se muestran los principales hongos y las toxinas que producen.

Tabla 1. Principales hongos y toxinas que producen

Hongo	Toxina
Aspergillus	Aflatoxinas (AFs) Ocratoxina A (OTA)
Claviceps	Alcaloides de la ergotamina
Fusarium	Fumonisina B1 (FB1) y B2 (FB2) Tricotecenos: deoxinivalenol (DON); nivalenol (NIV); Toxina T-2 (T-2), toxina HT-2 (HT-2) Zearalenona (ZEA) Micotoxinas emergentes Beauvericina (BEA); Eniatinas (ENs); Moniliformina (MON); Fusaproliferina
Penicillium	Citrinina (CIT) Patulina (PAT) Ocratoxina (OT)

No todos los hongos son toxigénicos, ni todas las micotoxinas se producen únicamente por un tipo de hongo. Algunas micotoxinas son producidas por una especie de hongo, mientras que otras son producidas por una amplia variedad de especies (Jard y col. 2011).

Las micotoxinas están presentes en los alimentos y los piensos, esto hace que supongan un riesgo para la salud humana y animal y que se produzcan importantes pérdidas económicas (Smith y col. 2016)

Las micotoxinas representan uno de los grupos más importantes de toxinas producidas biológicamente, penetran en el organismo con la ingestión de alimentos, bien directamente por el consumo de alimentos contaminados como cereales, frutas, etc. o bien indirectamente por el consumo de alimentos de origen animal como la leche, los huevos etc., preparados u obtenidos de animales que han sido alimentados con piensos contaminados. Las micotoxinas también ingresan en el organismo por la inhalación de esporas toxigénicas y por el contacto con la piel (Marin y col. 2013; Flores-Flores y col. 2015).

La producción de micotoxinas a veces es inevitable y depende de factores ambientales como la temperatura, la humedad y la actividad del agua y de otros factores como las malas prácticas agrícolas, las condiciones durante la cosecha, secado, manipulación, empaquetado, almacenamiento y transporte de alimentos. La contaminación con micotoxinas tiene lugar en varios puntos de la cadena alimentaria. (Marin y col. 2013; Stoev 2015).

Las micotoxinas se dividen en micotoxinas de campo que son las que se producen antes o inmediatamente después de la cosecha. Estas son principalmente producidas por el género *Fusarium.* Las micotoxinas de almacenamiento se producen durante el almacenamiento y el secado principalmente por los géneros *Aspergillus* y *Penicillium* (Smith y col. 2016).

Según la FAO (Organización de las Naciones Unidas para la Alimentación y la Agricultura), aproximadamente el 25% de la producción mundial de alimentos, está contaminada con al menos una micotoxina, lo

que causa importantes pérdidas económicas y efectos sobre la salud de humanos y animales. Debido a ello, es importante prevenir la contaminación con estrategias complementarias de descontaminación, que reduzcan la formación de micotoxinas ya que, la completa eliminación de las micotoxinas no es posible (Kolosova y Stroka 2011).

Las micotoxinas son activas a concentraciones bajas (Luch 2010), tienen un peso molecular bajo y diferentes estructuras químicas por lo que presentan un amplio rango de efectos tóxicos. Entre dichos efectos se incluye toxicidad pulmonar, neurotoxicidad, hemototoxicidad, toxicidad renal, problemas gastrointestinales, carcinogénesis, teratogénesis, inmunotoxicidad y efectos estrogénicos (Fung y Clark 2004; Van Egmond y col. 2007; Marin y col. 2010; Cortinovis y col. 2013; Escrivá y col. 2015; Dąbrowski y col. 2016).

La toxicidad de las micotoxinas depende de la concentración, el género de hongo y la especie que las producen, la edad del individuo, la dieta, el estado fisiológico y la duración de la exposición (Zielonka y col. 2015).

1.2. MICOTOXINAS DE *FUSARIUM*

Las especies del género *Fusarium* son los hongos más frecuentes en todo el mundo. Estos hongos producen una amplia gama de micotoxinas (Pizzo y col. 2016; Savard y col. 2016).

Los hongos del género *Fusarium* precisan climas templados para su crecimiento y la producción de micotoxinas. Estos invaden cereales importantes en la agricultura de América, Europa y Asia. Las micotoxinas más importantes del género *Fusarium* y las especies que las producen se detallan en la Tabla 2. Estas micotoxinas, junto con las aflatoxinas (AFs) del genero *Aspergillus* son las mayoritarias en los productos agrícolas. Se suelen encontrar en combinación con otras micotoxinas tanto en alimentos

de consumo humano como en piensos (Marin y col. 2013; Antonissen y col. 2014; Escrivá y col. 2015; Sun y col. 2015).

Tabla 2. Algunas de las principales micotoxinas producidas por especies del género *Fusarium* y la toxicidad que producen.

Micotoxina	Especies Productoras	Toxicidad
DON	*F. graminearum* *F. culmorum* *F. cerealis*	Alteraciones intestinales Alteraciones endocrinas Inmunotoxicidad
NIV	*F. gramicearum* *F. crookwellense* *F. nivale*	Inmunotoxicidad Alteraciones gastrointestinales
FB1	*F. monliforme, F.prolifratume, F. nigamai*	Nefrotoxicidad Hepatotoxicidad IARC: Grupo 2B
T-2	*F. sporotrichioides* *F. langsethiae* *F.acuminatum* *F. poae*	Hematotoxicidad Hepatotoxicidad Inmunotoxicidad Necrosis
ZEA	*F. gramiceaum* *F. culmorum* *F. cereales F.equiseti*	Disruptor endocrino Hepatotoxicidad Inmunotoxicidad Nefrotoxicidad
BEA	*F. Avenaceum* *F. poa* *F. proliferatum* *F. sambucinum* *F. langsethiae*	Inmunotoxicidad Apoptosis
ENs	*F. zvenaceum* *F. poae* *F. sporotrichioides F. tricictum*	Inmunotoxicidad Necrosis

Nota: BEA: beauvericina; DON: deoxinivalenol; ENs; eniatinas; FB1: fumonisina B1; IARC: Agencia Internacional de Investigación sobre el cáncer; NIV: nivalenol; T-2: Toxina T2; ZEA: zearalenona.

Teniendo en cuenta que las micotoxinas se producen simultáneamente en un mismo producto, es muy probable que los humanos y los animales estén expuestos a mezclas y no a una sola micotoxina a través de la dieta (Heussner y col 2006; Marin y col. 2013; Antonissen y col. 2014; Escriva y col. 2015; Sun y col. 2015). Debido a ello, hay un aumento sobre la preocupación del peligro que las micotoxinas suponen en la salud humana, ya que se pueden producir interacciones que aumente su toxicidad (Ruiz y col. 2011a; Ruiz y col. 2011b; Lu y col. 2013; Wan y col. 2013; Prosperini y col. 2014; Sun y col. 2015; Fernandez-Blanco y col. 2016).

1.3. ZEARALENONA Y METABOLITOS

La zearalenona (ZEA) es una micotoxina estrogénica producida por hongos del género *Fusarium* (Tabla 2). Es una lactona del ácido resorcíclico fusionada a un carbono macrocíclico el cual contiene un grupo metilo, grupo ceto y un doble enlace en configuración *trans* (Figura 1). El nombre de la ZEA viene de la combinación de diferentes elementos ZEA de *Gibberella zeae* o *Fusarium graminearum*, RAL por la lactona del ácido resorcíclico, EN por el doble enlace y ONE por el grupo ceto (Zinedine y col. 2007; Metzler y col. 2010). La ZEA tiene una estructura análoga a la de los estrógenos naturales como el 17β-estradiol, por lo que, es capaz de imitar la actividad de los estrógenos naturales compitiendo por la unión a los receptores estrogénicos (REs) (Fleck y col. 2012; Drzymala y col. 2015; Frizell y col. 2015).

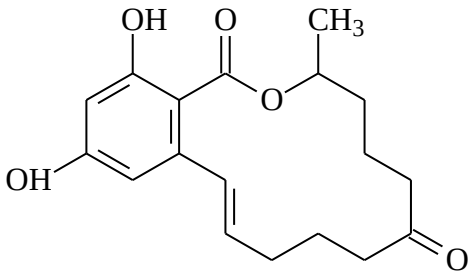

Figura 1. Estructura química de la zearalenona

La ZEA y sus metabolitos se producen durante el cultivo de cereales sobre todo el maíz pero también se encuentra en trigo, avena y cebada así como en productos derivados de los cereales como la harina y la cerveza (Zinedine y col. 2007). Se producen grandes cantidades de ZEA durante el almacenamiento de los cereales en condiciones de temperatura y humedad elevadas. Las condiciones ambientales durante la cosecha contribuyen a la producción de hongos del género *Fusarium*. La ZEA es termorresistente hasta 150ºC, resistente al almacenamiento, la molienda, el procesamiento de los alimentos y su cocción (Stadnik y col. 2010).

1.3.1. Propiedades biológicas

La ZEA controla el desarrollo de las plantas, siendo capaz de regular la floración debido a que tiene una actividad similar a la de las hormonas de las plantas (Meng y col. 1992; Biesaga-Koscielniak y Filek, 2010). Biesaga-Koscielniak (2001) demostraron que si se añadía ZEA a gérmenes del trigo aumentaba el porcentaje de plantas generativas. Biesaga-Koscielniak y col. (2003) demostraron que también se observa crecimiento de los gérmenes haploides del trigo. Además Koscielniak (2011) demostró que protegía al sistema fotosintético de la fuerte luz del sol.

1.3.2. Toxicocinética y mecanismo de acción

Se ha demostrado que las biotransformaciones de la ZEA ocurren en hongos, plantas y mamíferos, en la que están implicados el anillo aromático y el grupo alifático (Metzler y col. 2010).

Estudios realizados sobre la toxicocinética y metabolismo de la ZEA indican que esta micotoxina se absorbe rápidamente y puede ser metabolizada por el hígado principalmente, a α-zearalenol (α-ZOL) y β-zearalenol (β-ZOL) por la reducción del grupo cetona del C7. En la Figura 2 se muestran las reacciones de biotransformación de la ZEA en sus metabolitos y las estructuras del α-zearalanol (α-ZAL) y β-zearalanol (β-ZAL). Estas reacciones están catalizadas por las enzimas 3α-hidroxiesteroide deshidrogenasa y la 3β-hidroxiesteroide deshidrogenasa respectivamente. La proporción entre α-ZOL y β-ZOL varía dependiendo de la especie animal. Tanto la ZEA como los metabolitos generados se conjugan posteriormente con el ácido glucurónico a través de la enzima uridindifosfato-glucorinil transferasa (UGT). Los metabolitos generados, α-ZOL y β-ZOL se pueden reducir en menor cantidad a α-ZAL y β-ZAL por la reducción del doble enlace entre el C11 y el C12, aunque no se sabe con seguridad si estos metabolitos son producidos por los hongos del genero *Fusarium* o por reducción de la ZEA en los animales. Aunque la transformación *in vivo* ocurre principalmente en el hígado, otros órganos entre los que se incluyen pulmón, riñón e intestino, pueden contribuir al conjunto de las biotransformación (Schaut y col. 2008; Videmann y col. 2008; Metzler y col. 2010; Drzymala y col. 2015; Frizzell y col. 2015).

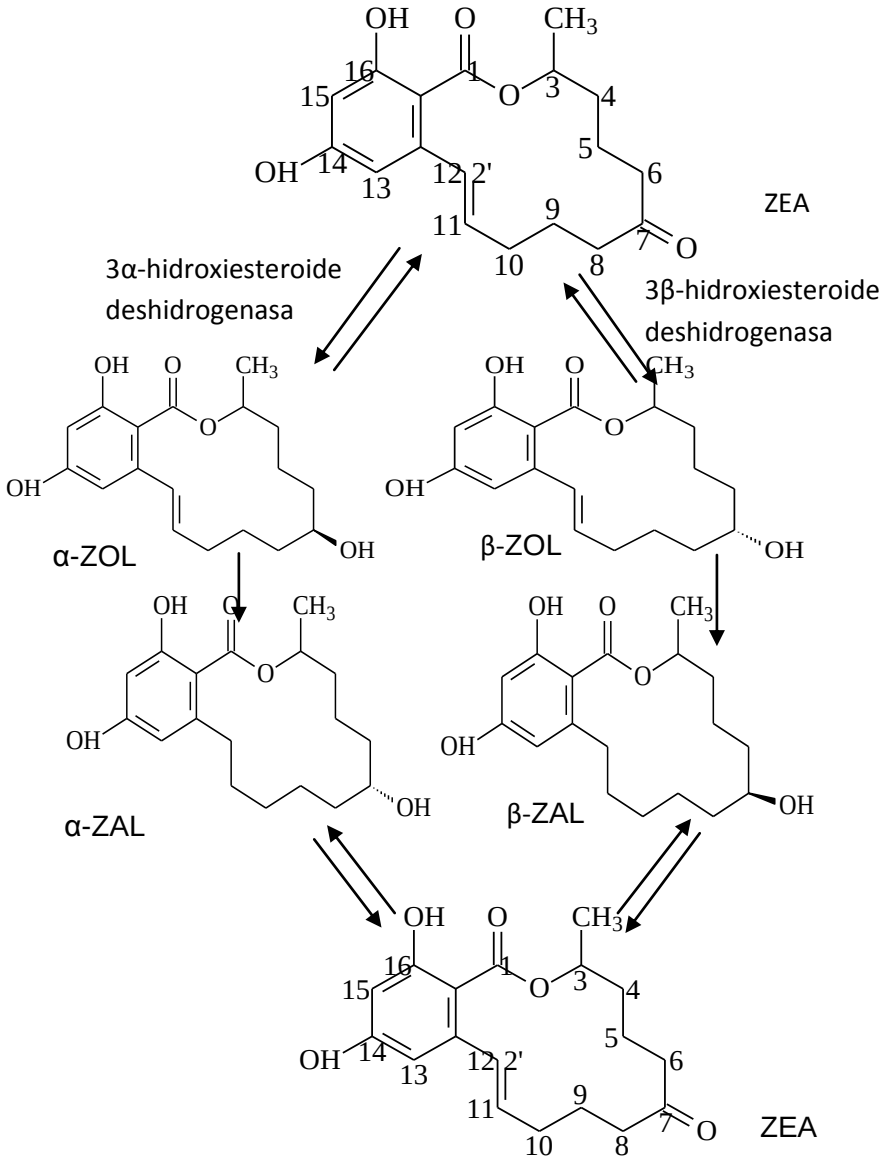

Figura 2. Vías de transformación de la ZEA en sus metabolitos mayoritarios

Tanto la ZEA como sus metabolitos ejercen su actividad por su unión a los REs. El complejo formado por la unión de la ZEA o sus metabolitos con

el RE se transfiere al núcleo celular donde se une con elementos responsables de la respuesta estrogénica provocando la transcripción de genes que median muchos de los efectos biológicos producidos por los estrógenos (Fink-Gremmels y Malekinejad, 2007). Los REs están formados por tres dominios: el dominio de unión C-terminal (LBD), el dominio central de unión al ADN (DBD) y el dominio N-terminal (NTD). En la Figura 3 se muestra la estructura del RE. En los humanos se han identificado dos isoformas diferentes de RE la α (REα) y la β (REβ). Los REα y REβ son homólogos en el dominio de unión al ADN en >90% de los aminoácidos y en el dominio C-terminal en un 55% (Kuiper y col. 1997). Varios estudios con cultivos celulares han demostrado que la ZEA es totalmente agonista de los REα mientras que para los REβ actúa como parcialmente agonista (Fink-Gremmels y Malekinejad 2007).

Figura 3. Estructura del receptor estrogénico. A, B y F son las zonas de la activación de la transcripción, la C es la zona de unión al ADN, la D la localización nuclear y la G es la zona de unión al ligando. N-: extremo N terminal; -c: extremo c terminal. DBD: el dominio central de unión al ADN; LBD: el dominio de unión C-terminal; NTD: el dominio N-terminal.

Las especies animales tienen una sensibilidad diferente a la ZEA dependiendo de la proporción de α-ZOL y β-ZOL que producen, siendo las especies más sensibles las que forman una mayor cantidad de α-ZOL (Schaut y col. 2008). Malekinejad y col. (2006) demostraron que mientras que en los pollos hay una mayor concentración de β-ZOL, en los cerdos la

ZEA principalmente se metaboliza a α-ZOL siendo estos especialmente sensibles a la ZEA (Songsermsakul y col. 2011).

1.4. TOXICIDAD

El efecto estrogénico es el efecto tóxico más importante de la ZEA. Este efecto depende del tipo y de la cantidad de REs presentes en los diferentes tipos celulares (Pizzo y col. 2015). A pesar de su baja toxicidad aguda la ZEA produce hepatotoxicidad, hematotoxicidad, inmunotoxicidad, genotoxicidad y es teratogénica. Recientemente ha aumentado el interés por la ZEA y sus metabolitos debido a que hay evidencias que sugieren que juegan un papel importante en los tumores hormono dependientes (estrógenos) aumentando el riesgo de padecerlos a las personas expuestas a ZEA (Gromadzka y col. 2008; Belhassen y col. 2015).

El α-ZOL es un metabolito de la ZEA que se ha utilizado como estimulador del crecimiento anabólico en el ganado en EEUU y otros países como Canadá, Australia, Nueva Zelanda, Japón, Chile y México. Sin embargo el uso del α-ZOL como estimulador del crecimiento es controvertido ya que es capaz de imitar la acción de las hormonas propias del ganado. Por este motivo, su uso está prohibido en muchos países como los miembros de la Unión Europea (UE) (Yuri y col. 2006; Belhassen y col. 2015).

Los efectos tóxicos de la ZEA han sido ampliamente estudiados en animales. Sin embargo, sus metabolitos no han sido tan ampliamente estudiados. En la Tabla 3 se muestran los efectos tóxicos de la ZEA y sus metabolitos *in vivo*.

Tabla 3. Efectos tóxicos de la ZEA, α-ZOL y β-ZOL *in vivo*

Micotoxina	Animal	Dosis	Resultado	Referencia
ZEA	Cerdas multíparas durante o después de la gestación	25-100 mg/Kg pc	Celo constante, infertilidad, reducción de la talla de los fetos, malformación y pseudogestación	Chang y col. 1979
	Cerdas embarazadas de 22-112 semanas	0,2, 0,5 y 1 mg/Kg pc	Disminución de los folículos	Schoevers y col. 2012
	Cerdos jóvenes	250 mg/Kg comida	Inmunosupresión hepática	Pistol y col. 2014
	Cerdos	1,04 mg/Kg pc	Aumento del número y tamaño de los folículos	Dai y col. 2016
		33,2 µg/Kg pc	No hay variación en la respuesta inmune	Zieloga y col. 2010
	Perros	75 µg/Kg pc	Apoptosis	Stopa y col. 2016
	Ovejas	14,49-3,07 µg/Kg	Activación de la respuesta inmune	Kostro y col. 2012
	Ratones	5, 10, 20 mg/kg pc	Aberraciones cromosómicas	Ayed y col. 2011
		40 mg/Kg pc	Apoptosis en el tejido cardiaco	Salem y col. 2015a
		40 mg/Kg	Afecta la función cerebral	Ren y col. 2016
		40 mg/Kg pc	Disminución en la movilidad y número de espermatozoides	Long y col. 2017
α-ZOL	Ratones	0,1ml	Anomalías en el desarrollo de los testículos	Perez-Martinez y col. 1996
		5, 10, 20 mg/kg pc	Aberraciones cromosómicas	Ayed y col. 2011

Micotoxina	Animal	Dosis	Resultado	Referencia
	Ovejas	4,05-2,87 µg/Kg pc	Activación de la respuesta inmune	Kostro y col. 2012
β-ZOL	Ratones	5, 10, 20 mg/kg pc	Aberraciones cromosómicas	Ayed y col. 2011

Nota: pc: peso corporal, α-ZOL: α-zearalenol, β-ZOL: β-zearalenol, ZEA: zearalenona.

1.4.1. Toxicidad *in vitro*

La citoxicidad de las micotoxinas se puede determinar con métodos alternativos mediante cultivos celulares (Repetto 2009).

Los cultivos celulares siguen los principios de las tres R (3Rs), propuestos por Russel y Buch en 1959. Estos principios abordan estrategias que conducen a reducir, refinar y reemplazar el uso de animales de laboratorio sin comprometer la calidad de los resultados obtenidos. Las 3Rs se incorporan en las guías de ensayos de toxicidad desarrolladas por la Organización para la Cooperación Económica y el Desarrollo (OCED) y en las directrices para las pruebas reglamentarias aplicables para la evaluación de la seguridad y toxicidad de productos químicos, como la Agencia Europea de Productos Químicos (ECHA), la Autoridad Europea de Seguridad Alimentaria (EFSA), el Programa Internacional de Seguridad Química, Pesticidas y Cosméticos (IPCS), la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) y la Agencia Europea de Medicamentos (EMA) (Kandárová y Letašiova 2011; Törnqvist y col. 2014).

Con el fin de disminuir el uso de animales de experimentación, se han creado comités nacionales e internacionales para validar los métodos alternativos como el Centro Europeo para la Validación de Métodos Alternativos (ECVAM), esto supone un beneficio económico y mejora el punto de vista social sobre los ensayos de toxicidad (Abud y col. 2015).

Esto se recoge en la directiva de 2010/63/UE del parlamento Europeo y del consejo de 22 de septiembre de 2010 relativa a la protección de los animales utilizados para fines científicos (EUROPEO 2010).

Los ensayos *in vitro* se usan como pruebas complementarias o de criba a los ensayos con animales. Los métodos *in vitro* proporcionan una información más detallada sobre los mecanismos de acción tóxica a nivel celular (Repetto 2009).

Los procesos que se detectan por métodos *in vitro* son aquellos que se desarrollan a nivel celular como son la toxicidad basal, el daño celular directo, la modificación de la actividad enzimática, etc. La mayor ventaja en los ensayos de citotoxicidad es que se hace una detección inmediata de los mecanismos celulares producidos por cada dosis ensayada. Por lo tanto, estos ensayos sirven para la preselección de la dosis en los estudios posteriores (Ukelis y col. 2008).

El interés por desarrollar sistemas celulares para los ensayos de citotoxicidad ha ido aumentando, ya que son más rápidos de aplicar, más baratos, su mantenimiento es sencillo, son métodos más cuantitativos porque hay una gran cantidad de células homogéneas, tienen una elevada reproducibilidad y evitan los problemas éticos asociados a la experimentación *in vivo* (Gutleb y col. 2002). Además, el uso de un gran número de células por ensayo, permiten una disminución de la variabilidad entre los experimentos. Por tanto, los cultivos celulares se consideran sensibles y reproducibles para realizar un cribado de toxicidad de las micotoxinas (Wan y col. 2013).

1.4.1.1. Citotoxicidad individual

Los ensayos de citotoxicidad sirven para evaluar la capacidad de determinadas sustancias para producir daños en las células.

Entre los ensayos utilizados para determinar la viabilidad y proliferación celular se incluyen métodos del bromuro de 3[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio (MTT), del Rojo Neutro (RN), de la lactato deshidrogenasa (LHD), del azul de tripán, de la incorporación de 5-bromo-2´-deoxiuridina (BrdU), de la fluoresceína diacetato (FDA), de la sulforhodamina B (SRB) etc.

Estos diferentes ensayos miden diferentes funciones celulares, por lo tanto las respuestas observadas proporcionan información de los mecanismos de acción de las micotoxinas (Minervini y col 2004).

El método MTT fue introducido por Mosmann en 1983. Este método determina la capacidad de las células vivas para convertir la sal soluble de tetrazolio (amarilla) en cristales insolubles de formazán (morados) por las enzimas succinato deshidrogenasas mitocondriales que forman parte del ciclo del ácido cítrico y de la cadena respiratoria y que requieren NADPH, que proporcionan las células vivas (Cetin y Bullerman 2005). La cantidad de formazán formado es proporcional al número de células vivas. Además es un indicador de la integridad mitocondrial (Abid-Essefi y col. 2004). El método MTT se utiliza para conocer la actividad metabólica de las células. El método MTT muestra una mejor correlación con los ensayos *in vivo* que otros métodos utilizados para medir la citotoxicidad (Wan y col. 2013). Es rápido, versátil, cuantitativo y fácil de reproducir. El método WST es una modificación del método MTT en este método la sal de tatrazolium es soluble en agua (Lu y col. 2013b).

El método del RN se basa en que el colorante RN solo puede ser captado por las células vivas que lo incorporan a los lisosomas. El colorante que es débilmente catiónico, atraviesa mediante difusión pasiva a través de las membranas celulares y se une a grupos fosfatos y carboxílicos de la matriz de los lisosomas. Los xenobióticos que dañan los

lisosomas hacen que disminuya la absorción del RN y disminuye la retención del mismo, ya que sólo se retiene el colorante en las células vivas (Ferrer y col. 2009).

El método de la LDH mide la actividad de dicha enzima en el medio extracelular. La pérdida de la LHD intracelular y su liberación al medio de cultivo es un indicador de la muerte celular irreversible, debido al daño producido en la membrana celular. El método consiste en la reducción del lactato a piruvato debido a la presencia de la enzima LDH y del NADH. Esta reducción del lactato a piruvato produce un cambio en la absorbancia proporcional a la cantidad de células viables (Fotakis y Timbrell 2006; Venkataramana y col. 2014).

El azul de tripán es un colorante ácido. Las células viables excluyen los colorantes ácidos a través de la membrana plasmática, por lo tanto su retención es un indicador del daño irreversible en la membrana celular que precede a la muerte celular. Las células vivas no se tiñen y las muertas sí. El colorante azul tripán es muy utilizado, pero tiene el inconveniente de que también tiñe las proteínas solubles, por lo que se puede producir una disminución en la precisión si el medio contiene más de 1% de suero. La retención del colorante depende de su concentración y del pH. Por lo tanto se ha de tener en cuenta estos dos parámetros a la hora de interpretar los resultados (Masters 2000; Fornelli y col. 2004; Minervini y col. 2004; Lioi y col. 2004).

Monitorizar la síntesis de ADN es un buen método para determinar la proliferación celular. El BrdU es un nucleótido sintético análogo a la timidina. El método BrdU determina la viabilidad celular midiendo la síntesis del ADN. La incorporación del BrdU en lugar de la timidina en el ADN de las células tiene lugar durante la fase S del ciclo celular. La cantidad de BrdU incorporada se determina de forma inmunoquímica,

usando un anticuerpo monoclonal a BrdU y la unión se cuantifica usando sustratos cromogénicos. Este método es un método sensible, más rápido y sencillo que el tradicional ensayo en el que se incorpora [^{3}H] timidina radioactiva. (Goyarts y col. 2006; Diesing y col. 2011; Masters 2000; Sundstøl y col. 2004; Minervini y col. 2004).

En el método de la FDA, a las células se les añade fluoresceína diacetato que no tiene fluorescencia y esta se convierte en fluorescente cuando se adhiere por esterasas a las células vivas. Por lo tanto, la emisión de fluorescencia se asocia a las células vivas (Bouaziz y col. 2008; Bensassi y col. 2014; Salem y col. 2016).

El método de la SRB se basa en la habilidad de la SRB de unirse a los componentes de las proteínas de las células que han sido fijadas en las placas de cultivo por el ácido tricloroacético (TCA). La SRB es una aminoxantina rosa brillante con dos grupos sulfónicos que se unen a los residuos de amino ácidos básicos en condiciones de pH neutro. La cantidad de colorante obtenida es directamente proporcional a la masa celular (Vichai y Kirtikara 2006)

Tanto la ZEA como sus metabolitos (α-ZOL y β-ZOL) producen citotoxicidad en diferentes líneas celulares utilizando diferentes criterios de valoración. En la Tabla 4 se muestran los datos de citotoxicidad obtenidos usando diferentes líneas celulares y métodos.

Tabla 4. Ensayos para determinar la viabilidad y proliferación celular en diferentes líneas celulares expuestas a ZEA y sus metabolitos.

Línea Celular	Ensayo/Tiempo	IC_{50} (μM) /Micotoxina	Referencia
BRL 3A	MTT/24h	140/ZEA	Sun y col. 2015
Caco-2	MTT/24h	>200/ZEA	Videman y col. 2008
		43/ZEA	Gao y col. 2016

Línea Celular	Ensayo/Tiempo	IC_{50} (µM) /Micotoxina	Referencia
		47,26/α-ZOL	
	MTT/48h	313/ZEA	Cetin y Bullerman 2005
		20/ZEA; 80/α-ZOL; 60/β-ZOL	Abid-Essefi y col. 2009
		20,07/ZEA	Gao y col. 2016
		28,54/α-ZOL	
	MTT/72h	25/ZEA	Kouadio y col. 2005
		15/ZEA	Abid-Essefi y col. 2004
		137/ZEA	Cetin y Bullerman 2005
		11,62/ZEA	Gao y col. 2016
		22,90/α-ZOL	
	RN/72h	15/ZEA	Kouadio y col. 2005
		313/ZEA	Kouadio y col. 2007
Células sanguíneas de cerdo	[^{3}H]incorporación de timidina/72h	6,00 ± 1,5/α-ZOL	Luongo y col. 2008
CHO-K1	MTT/24h	313/ZEA	Cetin y Bullerman 2005
		79,4/ZEA	Ferrer y col. 2009
	RN/24h	108,76/ZEA	
	MTT/48h	313/ZEA	Cetin y Bullerman 2005
C5-O	MTT/48h	313/ZEA	Cetin y Bullerman 2005
	MTT/72h	75,5/ZEA	

Línea Celular	Ensayo/Tiempo	IC_{50} (µM) /Micotoxina	Referencia
HCT116	FDA/24h	60/ZEA	Bensassi y col. 2014
	Azul de metileno/48h	>100/ZEA	Abassi y col. 2016
HEK293	FDA/24h	150/α-ZOL 240/β-ZOL	Salem y col. 2016
	WST/24h	80/ZEA	Sang y col. 2016
HeLa	MTT/24h	60/ZEA; 65/α-ZOL; 100/β-ZOL	Ayed y col. 2011
HepG2	MTT/3h	270/ZEA	Hassen y col. 2007
	MTT/24h	180/ZEA	
		100/ZEA	Ayed-Boussema y col. 2008
		150/ZEA	Lee y col. 2013
		80/ZEA	Wang y col. 2014a
		30/α-ZOL	
		113/ZEA	He y col. 2015
	FDA/24h	110/ZEA	El Golli Bennour y col. 2009
		110/ZEA	Bouaziz y col. 2008
		268/ZEA	Gazzah y col. 2010
	MTT/48h	313/ZEA	Cetin y Bullerman 2005
		80/ZEA	Wang y col. 2014a
		30/α-ZOL	
	FDA/48h	80/ZEA	Bouaziz y col. 2008
	MTT/72h	313/ZEA	Cetin y Bullerman 2005
		80/ZEA	Wang y col. 2014a
		30/α-ZOL	
Jurka T	FDA/24h	51,75 ± 2,99/α-ZOL	Luongo y col. 2006
Linfocitos de bovinos	Azul de tripan/24h	>0,4/ZEA	Lioi y col. 2004

Línea Celular	Ensayo/Tiempo	IC_{50} (µM) /Micotoxina	Referencia
PMN	MTT/1h	73,4/ZEA; 59/α-ZOL; 56,8/β-ZOL	Marin y col. 2010
RAW264.7	WST	50/α-ZOL 25/β-ZOL	Lu y col. 2013b
SH-SY5Y	MTT/24h	50/ZEA	Venkataramana y col. 2014
Vero	MTT/24h	50/α-ZOL	Othmen,y col 2008
		84/β-ZOL	
		120/ZEA	El Golli y col. 2006
		7/ZEA	Ayed-Boussema y col. 2007
		>100/ZEA	Bouaziz y col. 2012
	MTT/72h	7/ZEA	Abid-Essefi y col 2004
V79	MTT/48h MTT/72h	313/ZEA 313/ZEA	Cetin y Bullerman 2005
	NR/48h	26/ZEA	Behm y col. 2012
		50/α-ZOL	

Nota: BRL3A: células de hígado de rata; Caco-2: células de adenocarcinoma de colon humano; CHO-K1: células epiteloides de ovario del hámster chino; C5-O: células de queratinocitos de ratón; FDA: fluoresceína diacetato; HCT116: células de carcinoma de colon; HeLa: células de carcinoma de cuello de útero humano; HEK293: células embrionarias de riñón; HepG2: células de hepatoma humano; Jurka T: células linfoblastoides humanas; MTT: bromuro de 3[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio; RN: rojo neutro; PMN: neutrófilos de cerdo; RAW 264.7: macrófagos; SH-SY5Y: células de neuroblastoma humano; V79:células de fibroblastos de pulmón de hámster chino; Vero: células de riñón de mono; WST: tetrazolium soluble en agua; ZEA: Zearalenona; α-ZOL: Zearalenol; β-ZOL: Zearalenol.

1.4.1.2. Citotoxicidad de micotoxinas en combinación

Se sabe que diferentes micotoxinas producidas por la misma o diferentes especies de hongos están presentes simultáneamente en los productos vegetales y también pueden estar simultáneamente en los alimentos y piensos dependiendo de las condiciones medioambientales y del sustrato (Lei y col. 2013). Los efectos de la combinación de diversas micotoxinas no se conocen por completo, ya que no existe demasiada información al respecto (Wan y col. 2013). Teniendo en cuenta que la ZEA se metaboliza rápidamente por vía hepática a sus dos metabolitos mayoritarios (α-ZOL y β-ZOL), las mezclas de ellos pueden estar presentes en los sistemas biológicos y esto puede suponer un peligro para la salud dependiendo del tipo de interacción que tenga lugar (Ayed y col. 2011).

En la tabla 5 se muestra estudios de combinación de cultivos celulares en los que intervienen la ZEA o sus metabolitos, así como el tipo de interacción que producen.

Tabla 5. Combinaciones de micotoxinas estando presente la ZEA y/o alguno de sus metabolitos, el tipo de línea celular utilizada por los indicadores de toxicidad determinados y tipo de interacción producido.

Micotoxinas	Línea celular	Concentración (µM)	Tiempo/Ensayo	Resultado	Referencia
ZEA + AFB1	PK-15	ZEA: 10 AFB1: 5	MTT/48h	Sinergismo	Lei y col. 2013
	Caco-2	ZEA: 7,5-30 AFM1: 2,5-10	MTT/24h	Antagonismo a adición	Gao y col. 2016
	Caco-2	FB1: 10 ZEA 5 o 10	RN/72h	Efecto aditivo	Kouadio y col. 2007
	HepG2 RAW 264.7	ZEA: 0,05-12 AFB1: 0,025-3	Azul alamar/48h	Antagonismo	Zhou y col. 2017
ZEA + FB1	Caco-2	FB1:10 ZEA:10	MTT/72h	Antagonismo	Kouadio y col. 2013
ZEA + DON	Caco-2	DON:10, 20 ZEA:10, 20	RN/72h	Efecto aditivo	Kouadio y col. 2007
	HCT116	DON: 100 ZEA: 40	MTT/24h	Antagonismo	Bensassi y col. 2014
	CFU-GM	DON: 0.04-0.1 ZEA:0.2-10	NE/14 días	Adición	Ficheux y col. 2012
	HepG2 RAW 264.7	ZEA: 0,05-12 DON: 0.0008-0.10	Azul alamar/48h	Sinergismo	Zhou y col. 2017
ZEA + T-2	Vero	ZEA: 0-100 T-2: 0-100	MTT24h	Sinergismo	Bouraziz y col 2012

Micotoxinas	Línea celular	Concentración (µM)	Tiempo/Ensayo	Resultado	Referencia
	CFU-GM	ZEA:0.2-10 T-2: 0.0005-0.0016	NE14 días	Adición	Ficheux y col 2012
ZEA + OTA	HepG2	ZEA: 0-100 OTA: 0-20	MTT/24h MTT/48h MTT/72h	Antagonismo	Wang y col. 2014a
	Caco-2	ZEA: 7,5-30 OTA: 3,75-15	MTT/24h	Antagonismo a sinergismo	Gao y col. 2016
ZEA + α-ZOL	HepG2	ZEA: 0-100 α-ZOL: 0-50	MTT/24h MTT/48h MTT/72h	Antagonismo Antagonismo a sinergismo Antagonismo	Wang y col. 2014a
	Caco-2	α-ZOL: 11,25-45 ZEA: 7,5-30	MTT/24h	Sinergismo a antagonismo	Gao y col. 2016
α-ZOL + FB1	Jurkat T	α-ZOL : 10-80 FB1: 20 y 40	incorporación de timidina/24h	Antagonismo	Luongo y col. 2006
	Células sanguíneas de cerdo	α-ZEA: 0,5-20 FB1: 0,5-80	incorporación de timidina/72h	Sinergismo	Luongo y col. 2008
α-ZOL + OTA	HepG2	α-ZOL: 0-50 OTA: 0-20	MTT/24h MTT/48h MTT/72h	Antagonismo	Wang y col. 2014a

Micotoxinas	Línea celular	Concentración (µM)	Tiempo/Ensayo	Resultado	Referencia
	Caco-2	α-ZOL: 11,25-45 OTA: 3,75-15	MTT/24h	Antagonismo a sinergismo	Gao y col. 2016
ZEA + α-ZOL + AFM1	Caco-2	α-ZOL: 11,25-45 ZEA: 7,5-30 AFM1: 2,5-10	MTT/24h	Antagonismo a sinergismo	Gao y col. 2016
ZEA+ α-ZOL + OTA	Caco-2	α-ZOL: 11,25-45 ZEA: 7,5-30 OTA: 3,75-15	MTT/24h	Antagonismo a sinergismo	Gao y col. 2016
	HepG2	ZEA: 0-100 α-ZOL: 0-50 OTA: 0-100	MTT/24h MTT/48h MTT/72h	Antagonismo	Wang y col. 2014a
ZEA + DON + FB1	Caco-2	ZEA:10, 20 DON: 10, 20 FB1: 10	RN/72h	Sinergismo	Kouadio y col. 2007
ZEA + AFB1 + DON	HepG2 RAW 264.7	ZEA: 0,05-12 AFB1: 0,025-3 DON: 0.0008-0.10	Azul alamar/48h	Sinergismo	Zhou y col. 2017
ZEA + AFM1 + OTA	Caco-2	ZEA: 7,5-30 AFM1: 2,5-10 OTA: 3,75-15	MTT/24h	Antagonismo a sinergismo	Gao y col. 2016
α-ZOL + AFM1 + OTA	Caco-2	α-ZOL: 11,25-45 AFM1: 2,5-10 OTA: 3,75-15	MTT/24h	Antagonismo a adición	Gao y col. 2016

Micotoxinas	Línea celular	Concentración (µM)	Tiempo/Ensayo	Resultado	Referencia
ZEA + α-ZOL + AFM1 + OTA	Caco-2	α-ZOL: 11,25-45 ZEA: 7,5-30 AFM1: 2,5-10 OTA: 3,75-15	MTT/24h	Antagonismo a sinergismo	Gao y col. 2016

Nota: AFB1: aflatoxina B1; AFM1: aflatoxina M1; Caco-2: células de adenocarcinoma de colon humano; CFU-GM: unidades formadoras de colonia: granulocitos y macrófagos; DON: deoxinivalenol; FB1: Fumonisina B1; HepG2: células del hepatoma humano; HCT116: células de carcinoma de colon; Jurkat T: células de linfoblastoides humanas; LDH: lactato deshidrogenasa; MTT: bromuro de 3[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio; NE: no especificado; NIV: Nivalenol; RN: rojo neutro; OTA: Ocratoxina A; PK15: células de epitelio de riñón de cerdo; RAW 264.7: macrófagos; T-2: toxina T-2; Vero: células de riñón de mono ZEA: Zearalenona; α-ZOL: Zearalenol.

1.4.2. Biotransformación de las micotoxinas

Las micotoxinas pueden biotransformarse y presentarse con su estructura química, acetilada, glusosilada, sulfatada, etc. Estos metabolitos de las micotoxinas surgen como consecuencia del metabolismo de la micotoxina original por las plantas o los animales o por el propio hongo (Versiloskis y col. 2011).

Las biotransformaciones de las micotoxinas por los animales son de fase I y fase II. En las reacciones de la fase I las micotoxinas son principalmente hidroxiladas por el citocromo p450 (CYP450) o bien hidrolizadas por diferentes enzimas. Las reacciones de fase II, llamadas reacciones de conjugación, son reacciones en las que las micotoxinas se unen mediante enlaces covalentes a grupos como los sulfatos o el ácido glucurónico (Wen y col. 2016).

El interés por el estudio de los derivados de las micotoxinas o micotoxinas enmascaradas se debe al aumento de manifestaciones clínicas debidas a la ingesta de alimentos contaminados por micotoxinas (Broekaert y col. 2015).

Las micotoxinas enmascaradas son derivados de los metabolitos de fase II. Generalmente estas micotoxinas no están en los estudios de evaluación del riesgo y sus efectos biológicos son todavía desconocidos. Sin embargo el interés por el conocimiento sobre los efectos que tienen sobre la salud está aumentando debido a que pueden estar presentes junto a sus precursores en los alimentos y los piensos. Estas micotoxinas enmascaradas pueden tener un efecto tóxico directo o generar otros metabolitos tras la ingestión, por lo que podrá aumentar la exposición real si se compara con la exposición estimada donde no se consideran estos derivados (Dall'Erta y col. 2013).

Durante mucho tiempo solo se conocían las formas reducidas y los glucurónicos de la ZEA. Posteriormente se determinaron otros metabolitos de la ZEA como son los derivados monohidroxilados o los sulfatados que son los principales derivados de la ZEA y sus metabolitos (Pfeiffer y col. 2011; Drzyala y col. 2015; De Boevre y col. 2015).

En la Tabla 6 se muestran los diferentes derivados de la ZEA y sus metabolitos.

Tabla 6. Derivados obtenidos tras procesos de biotransformación sufridos por la ZEA y sus principales metabolitos.

Micotoxina	Derivado	Referencia
ZEA	Diglucosido	Veršilovskis y col. 2011
	Glucósido	
	Gucurónido	Videmann y col. 2008 Pfeiffer y col. 2011
	Sulfato	Pfeiffer y col. 2011
	Hidróxido	Hildebrand y col. 2012
α-ZOL	Glucurónido	Videmann y col. 2008 Pfeiffer y col. 2011
	Sulfato	Pfeiffer y col. 2011
β-ZOL	Glucurónido Sulfato	Pfeiffer y col. 2011

Nota: ZEA: zearalenona; α-ZOL: α-zearalenol; β-ZOL: β-zearalenol.

1.4.3. Producción de especies reactivas de oxígeno (ROS): Estrés oxidativo

El estrés oxidativo es el desequilibrio entre los factores prooxidantes y los factores oxidantes a favor de los factores prooxidantes. La acumulación de especies reactivas de oxígeno (ROS) como el anión superóxido ($O^{-\bullet}_2$), el peróxido de hidrógeno (H_2O_2) o el radical hidroxilo (•OH) conduce al estrés oxidativo (Gomes y col. 2005; Dinu y col. 2011; Mike y col. 2013; Prosperini y col. 2013; Wang y col. 2014b).

Las mitocondrias son la principal fuente de producción de ROS (Zhu y col. 2012). La producción de ROS está involucrada en funciones fisiológicas importantes de los organismos aerobios. Los radicales libres tienen un papel muy importante en el origen de la vida y la evolución biológica, produciendo efectos beneficiosos sobre los organismos (Uttara y col. 2009). Las ROS están implicadas en muchas actividades bioquímicas de las células, tales como la traducción de señales, la transcripción de genes y la regulación de la actividad de la adenilato ciclasa soluble. De igual modo, intervienen en la regulación redox de la fosforilación de proteínas, canales iónicos y procesos de biosíntesis de hormonas tiroideas (Uttara y col. 2009; Brieger y col. 2012). Sin embargo una producción excesiva de ROS conduce a una alteración de las vías metabólicas, disminución de los antioxidantes celulares y provoca que se oxiden estructuras macromoleculares, entre las que se encuentra lípidos, proteínas y el ADN (Ferrer y col. 2009, Uttara y col. 2009, Brieger y col. 2012; Bouaziz y col. 2013).

Por tanto, la producción excesiva de ROS conduce a muchas enfermedades crónicas tales como la aterosclerosis, el cáncer, la diabetes, la artritis reumatoide, lesión de perfusión post-isquémica, infarto de

miocardio, enfermedades cardiovasculares, inflamación crónica, derrame cerebral y el shock séptico, en los seres humanos (Uttara y col. 2009).

Hay diferentes métodos para determinar la producción de ROS por métodos *in vitro* mediante cultivos celulares. Entre los que se incluyen la prueba de la 2',7'-diclorodihidrofluoresceína diacetato (H_2-DCFDA), el ensayo del nitroazul de tretazolium (NAT), el ensayo de la lucigenina (CL), el luminol, el ensayo del ferrocitocromo C, el ensayo de la cumarina, etc.

La prueba de la H_2-DCFDA es la más empleada. Se utiliza para monitorizar la producción de ROS a tiempo real. Es un método muy sensible (Ferrer y col. 2009; Eruslanov y Kusmartsev 2010). La oxidación del 2,7-diclorodihidrofluoresceína (DCFH) origina 2,7-diclorofluoresceína (DCF) que es un compuesto fluorescente. Inicialmente se pensó que solo era útil como un indicador específico para H_2O_2, sin embargo, se demostró que la DCFH se oxida por otras ROS, tales como HO• y ROO•. La forma H_2-DCFDA se puede aplicar en estudios de células debido a su capacidad de difundir a través de las membranas celulares, siendo hidrolizada por esterasas intracelulares a DCFH (Gomes y col. 2005). Este método es fácil de utilizar, es muy sensible a los cambios en las ROS formadas en las células, es económico y puede ser utilizado para ver los cambios de las ROS a lo largo del tiempo (Eruslanov y Kusmartsev 2010).

El ensayo del NAT es un ensayo semicuantitativo que se usa para determinar la producción de $O_2^{-\bullet}$. Este ensayo determina la actividad de la enzima superóxido dismutasa (SOD) total utilizando un sistema xantina/xantina oxidasa como regulador del radical $O_2^{-\bullet}$, el cual al contactar con el NAT lo reduce a formazán. Este cambio se detecta por espectrofotometría cuando la SOD inhibe la reducción del NAT. Sin embargo este ensayo es semicuantitativo y es propenso a sesgos del observador (Choi y col. 2006; Uy y col. 2011).

El ensayo de la CL es uno de los métodos más sensibles para detectar ROS. Sin embargo, la CL es un compuesto aromático incapaz de atravesar las membranas celulares por lo tanto solo se utiliza para detectar el ROS extracelular (Mahomoodally y col. 2012).

El luminol se utiliza para determinar ROS, ya que es capaz de pasar a través de las membranas celulares. Por lo tanto, puede detectar tanto el ROS intra- como extracelulares. El luminol es sometido a una oxidación de sus electrones generándose un endoperóxido inestable. La descomposición de este endoperóxido genera luminiscencia mediante la emisión de fotones (Mahomoodally y col. 2012).

El método de la reducción de ferricitocromo C a ferrocitocromo C en presencia de $O_2^{-\bullet}$ es otro método utilizado para determinar ROS (Cardenas y Pedraza 2005).

La cumarina es un compuesto no fluorescente, sin embargo la hidroxilación de estos compuestos por el $OH^{\bullet}$ genera compuestos fluorescentes. Por lo que se utiliza para determinar las ROS a nivel celular. La fluorescencia de cumarinas hidroxiladas depende en gran medida del sitio de hidroxilación del anillo aromático. Por lo tanto, para la cuantificación precisa del $HO^{\bullet}$, por lo que es esencial identificar los productos de hidroxilación de cumarina (Gomes y col. 2005).

Diferentes artículos de estudios tanto *in vivo* como *in vitro* muestran que la ZEA y sus metabolitos pueden producir citotoxicidad a través de la producción de ROS (Salem y col. 2017)

En la Tabla 7 se muestran diferentes métodos para determinar la producción de ROS por exposición a ZEA y sus metabolitos en diferentes líneas celulares.

Tabla 7. Producción de ROS en diferentes líneas expuestas a ZEA y sus metabolitos

Línea celular	Concentración (μM)/ Micotoxina	Ensayo/Tiempo	Producción de ROS (*)	Referencia
BRL3A	10-40 (ZEA)	H_2-DCFDA/24h	No aumenta	Sun y col. 2015
Caco-2	10 (ZEA)	H_2-DCFDA/24h	Aumento 5 veces	Kouadio y col. 2005
CHO-K1	1, 5, 50 (ZEA)	H_2-DCFDA/0min	Aumenta 4 veces	Ferrer y col. 2009
HCT116	100 (ZEA)	H_2-DCFDA/30min	Aumenta 2,57 veces	Salem y col. 2015b
HEK293	20 (ZEA)	H_2-DCFDA/2h	No hay aumento	Gao y col. 2013
	75 (ZEA)	H_2-DCFDA/30min	Aumenta 1,74 veces	Salem y col. 2015b
	20 (ZEA)	H_2-DCFDA/20min	Aumenta 1,12 veces	Sang y col. 2016
	40 (ZEA)	H_2-DCFDA/20min	Aumenta 1,37 veces	Sang y col. 2016
	150 (α-ZOL) 240 (β-ZOL)	H_2-DCFDA/30min	Aumenta 5,34 veces Aumenta 4,36 veces	Salem y col. 2016
HepG2	100 (ZEA)	H_2-DCFDA/60h	Aumenta 4 veces	Gazzah y col. 2010
	120 (ZEA)	H_2-DCFDA/20min	Aumenta 5 veces	Bouaziz y col. 2008
	140 (ZEA)	H_2-DCFDA/60min	Aumenta 3 veces	He y col. 2015
H9c2	50 (ZEA) 75 (α-ZOL) 80 (β-ZOL)	H_2-DCFDA/60min	Aumenta de 10 a 9 veces	Salem y col. 2017
MA-10	30 (ZEA)	H_2-DCFDA/1h	Aumenta 2 veces aproximadamente	Savard y col. 2016
macrófagos	50 (α-ZOL) 50 (β-ZOL)	H_2-DCFDA/30min	Aumenta 1.5 veces Aumenta 2 veces	Lu y col. 2013b

Línea celular	Concentración (µM)/ Micotoxina	Ensayo/Tiempo	Producción de ROS (*)	Referencia
MLTC-1	5-20 (ZEA)	H_2-DCFDA/20min	Aumenta 1.2-1.4 veces	Li y col. 2014
PK-15	10 (ZEA)	H_2-DCFDA/30min	No aumenta	Lei y col. 2013
PMNs	1 (β-ZOL)	NAT/1h	Aumenta	Marin y col. 2010
	1 (β-ZOL)	Citocromo C/20 min	Aumenta	
SH-SY5Y	200 (ZEA)	H_2-DCFDA/30min	Aumenta 3 veces	Venkatara mana y col. 2014
Vero	21,25-85(α-ZOL) 13,75-55 (β-ZOL)	H_2-DCFDA	Aumenta 5 veces Aumenta 7 veces	Othmen y col. 2008

Nota: BRL3A: células de hígado de rata; Caco-2: células de adenocarcinoma de colon humano; CHO-K1: células epiteloides de ovario del hámster chino; HCT116: células de carcinoma de colon; HEK293: células embrionarias de riñón; HepG2: células de hepatoma humano; H_2-DCFDA: 2',7'-diclorodihidrofluoresceína diacetato; H9c2: células cardiacas; MA-10: células tumorales de ratón; MLTC-1: células tumorales de ratón; NAT: nitroazul de tretazolium; PK-15: células de riñón de cerdo; PMNs: granulocitos; SH-SY5Y: células de neuroblastoma humano; Vero: células de riñón de mono; ZEA: Zearalenona; α-ZOL: Zearalenol; β-ZOL: β-Zearalenol (*): respecto al control.

1.4.4. Daño al ADN

Diversos agentes físicos o químicos, pueden producir daños directos o indirectos al ADN, bien adicionando determinados grupos al ADN o formando enlaces cruzados entre la doble hélice del ADN (Staneva y col. 2012).

En los casos donde no se haya podido reparar estas alteraciones del ADN o se replique el ADN con dicha alteración, pueden originarse mutaciones y alteraciones en los cromosomas produciendo la muerte celular (apoptosis o necrosis) o conducir a alteraciones heredadas genéticamente (Staneva y col. 2012; Guerard y col. 2014).

Existen diferentes ensayos para determinar el daño producido al ADN, como el ensayo del cometa, el de micronúcleos, la reacción en cadena de la polimerasa (PCR), etc.

El ensayo del cometa se ha convertido en uno de los métodos estándar para determinar el daño producido al ADN. Es un método que puede detectar y cuantificar rupturas en las cadenas simples y dobles del ADN, sitios lábiles alcalinos y procesos de reparación incompleta en células (Vigreux y col. 1998; Staneva y col. 2012). El ensayo del cometa es muy versátil y se puede aplicar en ensayos de biomotorización, epidemiología molecular, ecogenotoxicidad, y para la investigación básica sobre el daño producido al ADN y los procesos de reparación (Azqueta y Collins. 2013; Collins 2004a; Collins 2014b).

El ensayo del cometa tiene una serie de ventajas sobre otros métodos, es un método sensible que detecta pequeños daños en el ADN, se requiere pocas células por muestra, es un método flexible, de bajo coste, es fácil de aplicar y se requiere poco tiempo para realizar el experimento (Azqueta y Collins 2013; Kang y col. 2013)

El ensayo del cometa consiste en una combinación de una electroforesis en gel de agarosa con una microscopía fluorescente para visualizar la migración de los fragmentos de ADN de las células individuales. Si el ADN está fragmentado, los diferentes fragmentos pueden migrar al ánodo durante la electroforesis formándose una cola, si el ADN está en buen estado el gran tamaño del ADN impide que migre (Olive y Banáth 2006). En este ensayo se tienen que tener en cuenta una serie de factores que pueden alterar el resultado: la concentración de agarosa, el tiempo de incubación alcalina y el tiempo y voltaje de la electroforesis (Collins y col. 2014a).

La determinación de la cantidad de ADN que migra proporciona una forma sencilla de medir el número de roturas en el ADN en una célula (Olive y Banáth 2006). El porcentaje (%) de ADN en la cola puede resultar el parámetro más útil ya que se relaciona linealmente con el daño del ADN a través de una amplia gama de daños y con la frecuencia de ruptura. Por el contrario el momento en la cola no proporciona información sobre como es la cola y no tiene una relación linear entre el daño y la concentración (Lovell y Omori 2008). En la Figura 4 se muestra una célula con cometa.

Figura 4. Célula con daño en el ADN. Se muestra la migración de fragmento según el ensayo del cometa.

Otro método utilizado para determinar la genotoxicidad es el test de micronúcleos. Los micronúcleos son masas de cromatina que parecen pequeños núcleos y están presentes cerca del núcleo celular. Los micronúcleos se forman por la ruptura de los cromosomas (Corcuera y col. 2015). Este ensayo se usa para determinar la capacidad de una sustancia o micotoxina para producir alteraciones en los cromosomas. Sin embargo este ensayo es menos sensible en la evaluación del daño producido en el ADN (Van Goethen y col. 1997).

La reacción en cadena de la polimerasa (PCR) es uno de los métodos fiables más utilizados en la detección de los daños producidos al ADN, ya que detecta la replicación del ADN donde hay una rotura (Kumari y col. 2008).

Se ha visto que tanto la ZEA como los metabolitos pueden producir genotoxicidad mediante la inhibición de la síntesis de ADN, fragmentación del ADN, formación de micronúcleos, intercambio de cromátidas hermanas, aberraciones cromosómicas, etc.

En la Tabla 8 se muestra el daño producido por la ZEA y sus metabolitos en diferentes líneas celulares.

Tabla 8. Daño producido por la ZEA y sus metabolitos en el ADN.

Línea Celular	Concentración (μM)/Micotoxina	Ensayo/Tiempo	Daño al ADN	Control	Referencia
Caco-2	10 (ZEA) 20 (ZEA) 40 (ZEA)	Cometa/24h	3,42±20,62(MDA) 6,67±0,62(MDA) 8,13±2,07(MDA)	2,05±0,73	Abid-Essefi y col. 2009
	40 (α-ZOL) 80 (α-ZOL) 160 (α-ZOL)	Cometa/24h	3,57±0,29(MDA) 4,21±0,88(MDA) 6,11±0,25(MDA)	2,05±0,73	
	30 (β-ZOL) 60 (β-ZOL) 120 (β-ZOL)	Cometa/24h	5,35±0,59(MDA) 5,68±1,32(MDA) 6,94±0,61(MDA)	2,05±0,73	
CCL3	50-200 (ZEA)	Cometa/24h	7,43±0,35- 19,01±4,21(TM)	0,32±0,14	Kang y col. 2013
HCT116	100 (ZEA)	Cometa/24h	235,8±12,2(AU)	29±6	Salem y col. 2015b
HEK293	75 (ZEA)	Cometa/24h	198±11,57(AU)	19±6,3	Salem y col. 2015b
	2,5-20 (ZEA)	Cometa/2h	2,56±1,66- 14,64±4,28 (%)	2,27±1,17	Gao y col. 2013
HepG2	100 (ZEA)	Cometa/24h	112±2,1- 460±3,1(AU)	100	Gazzah y col. 2010
Linfocitos	15 (ZEA)	MN/48h	60 frecuencia MN	5	Ben Salah-Abbès y col. 2009
SH-SY5Y	25-100 (ZEA)	Cometa/NE	31±2,6-83±5,3 olive TM	10±1,5	Venkataramana y col. 2014
Vero	40 (ZEA) 60 (ZEA)	Cometa/24h	163±2,8(AU) 117±18,4(AU)	55±4	Abid-Essefi y col. 2012

Línea Celular	Concentración (µM)/Micotoxina	Ensayo/Tiempo	Daño al ADN	Control	Referencia
	120 (ZEA)		223,8±32,8(AU)		

Nota: AU: unidad arbitraria; Caco-2: células de adenocarcinoma de colon humano; CCL3: células de carcinoma del cuello uterino; Comet: ensayo del cometa; HCT116: células de carcinoma de colon; HEK293: células embrionarias de riñón; HepG2: células del hepatoma humano; MDA: producción de molondihaldehído; MLTC-1: células tumorales de ratón; MN: micronúcleos; NE: no especificado; TM: momento de cola; SH-SY5Y: células de neuroblastoma humano; Vero: células de riñón de mono; ZEA: Zearalenona; α-ZOL: Zearalenol

1.4.5. Sustancias con actividad estrogénica

Determinados compuestos químicos pueden imitar o antagonizar la acción *in vitro* o *in vivo* de los estrógenos naturales como el 17β-estradiol (E2). Estas sustancias se definen como sustancias con actividad estrogénica o sustancias con actividad antiestrogénica (Bittner y col. 2014). Las sustancias con actividad endocrina pueden interaccionar con diferentes componentes del sistema endocrino y está demostrado que las células son capaces de sintetizar hormonas esteroideas en cultivos celulares. Sin embargo estos cambios no tienen porque producirse en condiciones *in vivo* ya que hay mecanismos compensadores y regulatorios para el control hormonal (EFSA, 2013).

Muchos de estos compuestos tienen poca semejanza estructural obvia con el estrógeno natural pero son capaces de unirse a los RE, de influir en la expresión de genes regulados por los estrógenos, de regular el crecimiento de células dependientes de estrógenos y producen respuestas estrogénicas fisiológicas *in vivo* (Darbre y col. 2002).

Estas sustancias causan preocupación debido a que pueden interferir con el funcionamiento normal de los procesos endocrinos y por lo tanto puede interferir en la función hormonal en los humanos produciendo muchos problemas como pubertad precoz en hembras, reducción de espermatozoides, alteración de las funciones de los órganos reproductivos, obesidad, alteración de comportamientos sexuales y pueden aumentar algunos cánceres de mama, de ovarios, de testículos y de próstata (Bittner y col. 2014).

Los fetos, recién nacidos y mamíferos jóvenes son más sensibles a la actividad estrogénica y los efectos se han observado a dosis muy bajas. Estos efectos se pueden detectar por primera vez en la edad adulta. Muchos de estos efectos observados en otros mamíferos se espera que

también se produzcan en los humanos ya que los mecanismos básicos endocrinos se mantienen en gran proporción en diferentes especies de vertebrados (Bittner y col. 2014).

Hay un aumento de sustancias que simulan los efectos del estrógeno natural E2 produciendo una alteración de la actividad estrogénica (Korner y col. 1999). Se ha podido determinar la estructura de los RE de los humanos y se ha visto que el E2 natural cuando se une al receptor deja un espacio suficiente para que otras moléculas interaccionen con el RE produciendo una alteración en los niveles hormonales normales. (Brozozowski y col. 1997; Koner y col. 1999; Bicchi y col. 2009).

Hay diferentes métodos que se utilizan para la determinación de la actividad estrogénica como el E-Screen, la globulina de unión de hormonas sexuales (SHGB), el ensayo de la fosfatasa alcalina (ALP), el ensayo del gen reportero de la luciferasa (RGA), etc.

El ensayo del E-Screen se basa en las siguientes premisas: las moléculas presentes en el suero inhiben la proliferación de células sensibles a los estrógenos. Los estrógenos inducen la proliferación celular inhibiendo este efecto, pero los estrógenos no esteroideos y los factores de crecimiento no impiden que el suero produzca su efecto inhibitorio (Soto y col. 1995).

Este ensayo se realiza en células MCF-7 (células humanas cancerígenas de mama). En estas células se observa un aumento en la proliferación de las mismas debido a la unión de las sustancias a los RE que activan la producción hormonal. Esto es debido a que estas células tienen REα y se considera la línea celular más sensible para llevar a cabo el ensayo (Schilirò y col 2011, Lange y col 2014).

La SHGB es otro método que se basa en ser principal proteína de transporte para los estrógenos endógenos lipofílicos y andrógenos en el

suero sanguíneo ya que determinadas sustancias con actividad estrogénica son capaces de unirse a esta proteína produciendo una proliferación celular y la activación de los REs (Metzler y col. 2010).

En el ensayo de la ALP, los estrógenos estimulan la actividad de la enzima ALP, que se determina por medios fotométricos mediante la conversión del 4-nitrofenilfosfato a 4-nitrofenol (Lehmann y col. 2006).

El ensayo RGA se desarrolló para células de mamífero glandulares mediante la incorporación del gen de la luciferesa. En este ensayo a las células T47D se le transfiere el receptor androgénico humano (AR) y el gen de la luciferasa. Este es un método útil para detectar estrógenos en muestras ambientales. La actividad de la luciferasa se determina por la cuantificación de unidades relativas de luz (RLU) (Wehmas y col. 2011; Bermudez y col 2012). Las células H295R son utilizadas para observar los efectos endocrinos mediante la sobre- o infra- expresión de proteínas que son cuantificadas y cualificadas por la señalización de aminoácidos mediante isotopos en los cultivos celulares (SILAC), una electroforesis en gel y por espectrometría de masas (Busk y col. 2011). Estas células ya contienen los REα y REβ.

En la Tabla 9 se muestran diferentes resultados de la actividad estrogénica de la ZEA y sus metabolitos obtenidos por métodos diferentes.

Tabla 9. Actividad estrogénica de la ZEA y sus metabolitos.

Línea celular	Micotoxina	Ensayo	Resultado (*)	Referencia
HCT116	ZEA	RGA	400 PC (%)	Abassi y col. 2016
H295R	ZEA	RGA	0.9/PER (%)	Frizzell y col. 2011
	α-ZOL	RGA	68/PER (%)	

Línea celular	Micotoxina	Ensayo	Resultado (*)	Referencia
	β-ZOL	RGA	0,4/PER (%)	Frizzeli y col. 2011
MCF-7	ZEA	E-Screen	6/PE	Molina-Molina y col. 2014
	α-ZOL		6,1/PE	
	β-ZOL		5,9/PE	
	ZEA	E-Screen	112/RPE (%)	Minervini y col. 2005
	α-ZOL		103/RPE (%)	
	β-ZOL		112/RPE (%)	
	ZEA	SRB	250/PI	Parveen y col. 2009
	α-ZOL		340/PI	
	β-ZOL		200/PI	
	ZEA	RGA	8,7 10^{-10} EC_{50}	Demaegdt y col. 2016
	α-ZOL		3,1 10^{-11} EC_{50}	
	β-ZOL		1,3 10^{-8} EC_{50}	
Ovarios	α-ZOL	Contando células	31% PC	Pizzo y col. 2015

Nota: EC_{50}: concentración efectiva media; H295R: células de carcinoma adrenocortical humano; MCF-7: células de cáncer de mama; PC: proliferación celular; PE: efecto proliferativo; PER: potencial estrogénico relativo; PI: índice proliferativo; RGA: ensayo del gen reportero; SRB: ensayo de la sulforhamina B; ZEA: Zearalenona; α-ZOL: α-Zearalenol; β-ZOL: β-Zearalenol (*): respecto al control.

1.4.6. Sistemas de defensa celular

Las células tienen su propio mecanismo de protección antioxidante reduciendo antioxidantes endógenos que les ayudan a protegerse frente al efecto de las ROS. La defensa antioxidante está formado por enzimas tales como superóxido dismutasa (SOD), catalasa (CAT) y glutatión peroxidasa (GPx) y antioxidantes no enzimáticos tales como el glutatión (GSH). La disfunción de las enzimas antioxidantes está asociada con enfermedades como el Alzheimer, Huntington, Parkinson y la esclerosis lateral amiotrófica (Matés 2000).

El GSH es un tripéptido (L-gamma-glutamil-L-cisteinilglicina) y el antioxidante celular endógeno más importante ya que es uno de los compuestos más abundantes de las células que contienen grupos sulfihídrilo (Deponte y col. 2013). En la Figura 5 se muestra la estructura química del glutatión.

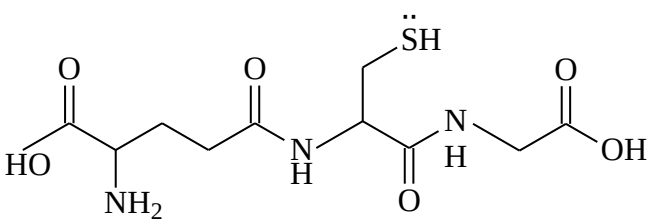

Figura 5: Estructura química del glutatión

En el GSH son muy importante las enzimas asociadas tales como GPx o la Glutatión reductasa (GR) (Deponte 2013). El glutatión oxidado (GSSG) reacciona con el NADPH por medio de la GR formando dos moléculas de GSH. El GSH neutraliza el H_2O_2 formando H_2O y GSH vuelve a su forma oxidada. Esta reacción esta catalizada por la GPx (Deponte 2013, Amir Aslami y Ghobadi 2016). Por lo tanto la habilidad de las células de reducir

o sintetizar el GSH es esencial para determinar la eficacia con la que las células son capaces de regular el estrés oxidativo (Matés 2000).

En la Figura 6 se muestra el esquema de las reacciones del GSH. En las que se muestra cómo el GSSG se transforma en la forma activa del GSH y como se oxida otra vez tras reaccionar con el H_2O_2.

$$GS\text{-}SG + 2H^+ + NADPH \xrightarrow{GR} NADP^+ + 2\ G\text{-}SH$$

$$2\ G\text{-}SH + H_2O_2 \xrightarrow{GPx} GS\text{-}SG + 2H_2O$$

Figura 6. Esquema de las reacciones del GSH con las enzimas GR y GPx.

La SOD es la única enzima capaz de dismutar el $O^{-\bullet}_2$ transformándolo en O_2 y H_2O_2. La SOD protege a las dehidratasas frente a la inactivación por los radicales superóxido. Esta enzima trabaja junto a la CAT y la GPx, neutralizando el H_2O_2 transformándolo en H_2O y O_2. Aunque la GPx comparte el sustrato H_2O_2 con la CAT puede reaccionar con los lípidos y otros peptidos orgánicos. (Matés 2000, Amir Aslami y Ghobadi 2016). En la Figura 7 se muestra el mecanismo de defensa de la SOD, CAT, GPx y GSH frente a las ROS

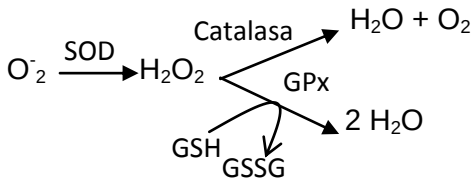

Figura 7. Mecanismo de defensa de las enzimas antioxidantes y del GSH frente a las ROS

En la Tabla 10 se muestra la alteración de la actividad de las diferentes enzimas antioxidantes y del sistema del GSH en diferentes líneas celulares cuando son expuestas a la ZEA. No se encuentran datos en la bibliografía consultada de los efectos de los metabolitos de la ZEA respecto al GSH y las enzimas antioxidantes.

Tabla 10. Efectos de las enzimas antioxidantes y del GSH en diferentes líneas celulares expuestas a la ZEA.

Línea celular	Concentración (µM)/Micotoxina	Enzima /GSH	Resultado (*)	Referencia
CCL3	200 (ZEA)	GSH	Aumenta	Lee y col. 2013
Granulocitos	15, 30, 60 (ZEA)	CAT SOD GPx GSH	Disminuye Disminuye Disminuye Disminuye	Qin y col. 2015
HepG2	200 (ZEA)	GSH	Disminuye	Lee y col. 2013
	3 (ZEA)	GSH	Disminuye	Lai y col. 2015
	200 (ZEA)	GSH	Aumenta	Hassen y col. 2007
Vero	40, 60, 120 (ZEA)	CAT	Aumenta	Abid-Essefi y col. 2012

Nota: CAT: catalasa; CCL3: células de carcinoma del cuello uterino; GSH: glutatión; HepG2: células del hepatoma humano; Vero: células de riñón de mono (*): respecto al control.

1.5. BIBLIOGRAFIA

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OBJETIVOS

2. OBJETIVOS

El objetivo general de la presente tesis doctoral es evaluar y comparar los efectos producidos por la ZEA y sus principales metabolitos (α-ZOL y β-ZOL) en diferentes células de mamífero. Para lograr este objetivo, se proponen los siguientes objetivos específicos:

1. Determinar y comparar la citotoxicidad de la zearalenona, α-zearalenol y el β-zearalenol en las células de ovario de hámster chino (CHO-K1) y en las células de hepatoma humano (HepG2) tras 24, 48 y 72 h de exposición mediante el ensayo MTT.
2. Determinar y comparar el tipo de interacción de las combinaciones de zearalenona, α-zearalenol y β-zearalenol en células CHO-K1 y HepG2, aplicando el método de las isobolas.
3. Evaluar y comparar la degradación de la zearalenona y sus metabolitos α-zearalenol y β-zearalenol en las células CHO-K1 y HepG2.
4. Determinar y comparar el estrés oxidativo mediante la producción de especies reactivas de oxígeno tras exposición a zearalenona, α-zearalenol y β-zearalenol en las células CHO-K1 y HepG2.
5. Determinar y comparar el daño al ADN producido por la zearalenona y sus metabolitos en las células CHO-K1 y HepG2.
6. Determinar el efecto de la disrupción endocrina que produce la zearalenona y sus metabolitos en las células MCF-7.
7. Determinar y comparar la actividad de las enzimas antioxidantes superóxido dismutasa (SOD), catalasa (CAT) y glutatión peroxidasa (GPx) y el sistema antioxidante no enzimático glutatión (GSH) en células CHO-K1 y HepG2 expuestas a ZEA y sus metabolitos.

RESULTADOS

3. RESULTADOS

3.1 Interactive effects of zearalenone and its metabolites on proliferation and metabolization in ovarian (CHO-K1) cells

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Interactive effects of zearalenone and its metabolites on proliferation and metabolization in ovarian (CHO-K1) cells

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Abstract

Zearalenone (ZEA) is a non-steroidal estrogen mycotoxin with high binding affinity to estrogen receptors. ZEA is rapidly absorbed and metabolized *in vivo* to α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). So, mixtures of them may be present in biological systems and suppose a hazard to animals and human health. The aims of this study were to determine the cytotoxic effects of ZEA and its metabolites, alone and in combination in ovarian (CHO-K1) cells during 24, 48 and 72 h by the MTT assay; and to investigate the metabolism of the CHO-K1 cells on ZEA, and its conversion into α-ZOL and β-ZOL by CHO-K1 cell after 24 and 48 h of exposure. The IC_{50} value obtained for individual mycotoxins range from 60.3 to >100.0 μM, from 30.0 to 33.0 μM and from 55.0 to >75.0 μM for ZEA, α-ZOL and β-ZOL, respectively. Cytotoxic interactions were assayed by the isobologram method, which provides a combination index (CI) value as a quantitative measure of the degree of the three mycotoxin interaction. The CI values for

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binary combinations ranged from 0.56 ± 0.15 (synergism at low concentrations) to 5.25 ± 5.10 (addition at high concentrations) and tertiary combinations from 2.95 ± 0.75 (antagonism at low concentrations) to 0.41 ± 0.23 (synergism at high concentrations). The concentration of ZEA and its metabolites was determined with liquid chromatography coupled to the mass spectrometer detector-linear ion trap (LC–MS–LIT). The percentage of ZEA degradation ranged from 4% (24 h) to 81% (48 h). In the same conditions, α-ZOL and β-ZOL concentration decreased from 8% to 85%. No conversion of ZEA in α-ZOL and β-ZOL was detected. However, at 24 h of exposure other degradation products of ZEA and its derived were detected.

Keywords: Zearalenone; Metabolites; Cytotoxicity; Interactions; LC–MS–LIT

1. Introduction

Mycotoxins are secondary metabolites of fungi and are present all over the world in food and feed, which suppose large varying levels of risk factor for human and animal health (Luongo et al., 2006). Zearalenone (ZEA) is a non-steroid estrogen *Fusarium* mycotoxin produced by numerous field fungi that grow in temperate areas of America, Europe and Asia. It exists worldwide contaminate cereal grains such as: maize, wheat, oats, soybeans (Luongo et al., 2006, Zinedine et al., 2007 and Marin et al., 2010).

ZEA is rapidly absorbed and biotransformed in the liver to α-Zearalenol (α-ZOL) and β-Zearalenol (β-ZOL) and, these metabolites can be reduced to α-Zearalanol (α-ZAL) and β-Zearalenol (β-ZAL), respectively. ZEA and

its reduced metabolites can be conjugated with glucoronic acid which facilitate their elimination (Zinedine et al., 2007 and Videmann et al., 2008).

ZEA and its metabolites have been shown to bind competitively to estrogen receptors (ERα and ERβ) due to have sufficient structural similarity to the endogenous estrogen, the 17β-oestradiol (Kouadio et al., 2007, Metzler et al., 2010 and Bouaziz et al., 2012). They produce estrogen gene activation and cause alterations in the reproductive system, that is, reproductive disorders. They also produce hematotoxicity, cytotoxicity, genotoxicity (DNA adduct formation, DNA fragmentation), immunotoxicity and hepatotoxicity. Moreover, ZEA is classified under group three carcinogens by the IARC (Luongo et al., 2006, Kouadio et al., 2007, Ferrer et al., 2009, Marin et al., 2010 and Prouillac et al., 2012).

The ZEA can metabolize in α-ZOL and β-ZOL which represent a serious hazard to animals and human health. Moreover, the resulting effect of a combined exposure can produce: synergism, antagonism and additive effect. In spite of cytotoxicity of ZEA and its metabolites alone has been widely studied, there are few studies about cytotoxicity produced by their mixtures (Kouadio et al., 2007, Luongo et al., 2006, Luongo et al., 2008 and Bouaziz et al., 2012). In order to determine the cytotoxic effect of ZEA and its metabolites, the aims of this study were (a) to determine the cytotoxic effects of ZEA and its metabolites, alone and in combination in ovarian (CHO-K1) cells, and analyze if the mixtures of these mycotoxins produced synergies, antagonism or additive effect and (b) to investigate the metabolism of the CHO-K1 cells on ZEA, and its conversion into α-ZOL and β-ZOL by CHO-K1 cells.

2. Materials and methods

2.1. Reagents

All reagents and cell culture components were of standard laboratory grade from Sigma–Aldrich (St. Louis Mo. USA). Standard of ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol) were purchased from Sigma–Aldrich (St. Louis Mo. USA). Acetonitrile and methanol were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). Stock solutions of mycotoxins were prepared in methanol and maintained at −20 °C in the dark. The final methanol concentration in the medium was 1% (v/v).

2.2. Cell culture and treatment

CHO-K1 cells were grown at 37 °C in 9 cm^2 polystyrene tissue culture dishes with Ham's-F12 medium supplemented with 25 mM HEPES buffer (pH 7.4), 10% fetal calf serum (FCS, from Cambrex Company, Belgium), 100 U/ml penicillin and 100 mg/ml streptomycin. The incubation conditions were 5% CO_2 in 37 °C and 95% air atmosphere at constant humidity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma–Aldrich, St. Louis Mo. USA).

2.3. Determination of cytotoxicity assays

CHO-K1 cells were plated in 96-well microplates at a density of 2×10^4 cells/well. After cells reached 65% confluence, the culture medium

was replaced with fresh medium containing the following concentrations of each mycotoxin: ZEA (12.5, 18.75, 25, 37.5, 50, 75 and 100 μM), α-ZOL (6.5, 9.37, 12.5, 18.75, 25, 37.5, 50, 75 and 100 μM) and β-ZOL (6.5, 9.37, 12.5, 18.75, 25, 37.5, 50 and 75 μM). Cytotoxicity was determined by the MTT assay performed as described by Ruiz et al. (2006). After 24, 48 and 72 h, the medium was removed and cells of each well received fresh medium containing 50 μl MTT. After 4 h of incubation at 37 °C under darkness, the resulting formazan was solubilized in DMSO. The absorbance was measured at 570 nm using an automatic ELISA plate reader (MultisKanEX, Labsystem, Helsinki, Finland).

2.4. Experimental design and assessment of effect of mycotoxin combinations: isobolograms method

CHO-K1 cells were exposed to several dilutions of each binary and tertiary mycotoxin combination. These mixtures were prepared as follows: ZEA + α-ZOL and ZEA + β-ZOL at constant 2:1 ratio, α-ZOL + β-ZOL at constant 1:1 ratio and ZEA + α-ZOL + β-ZOL at constant 2:1:1 ratio. Five dilutions of each mycotoxin combination (ZEA 12.5, 18.75, 25, 37.5 and 50 μM; α-ZOL and β-ZOL: 6.25, 9.37, 12.5, 18.75 and 25 μM) plus a control were tested in three independent experiments. CHO-K1 cells treated with 1% of methanol in the culture medium were considered as control. Cytotoxicity of combinations was determined by the MTT assay as described previously (Ruiz et al., 2006).

The isobologram analysis was used to determine the type of interaction that occurs when ZEA and its metabolites are in combination. The median-effect/combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984) was originally for analyzing drug combination effects. The isobologram analysis involves plotting the dose-

effect curves for each compound and its combinations in multiple diluted concentrations by using the median-effect equation:

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

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

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

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

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

$$CI=D_1/(D_x)_1+D_2/(D_x)_2$$

$$D_x=D_m[fa/(1-fa)]^{1/m}$$

$$CI=(D)_1/(D_m)_1[fa/(1-fa)]^{1/m1}+(D)_2/(D_m)_2[fa/(1-fa)]^{1/m2}$$

where in the denominator $(D_x)_1$ is for D_1 ‘‘alone’’ that inhibits a system x%, and $(D_x)_2$ is for D_2 ‘‘alone’’ that inhibits a system x%.

And, the general equation for *n*-compound combination at *x*% inhibition becomes:

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

where $^{n}(CI)_x$ is the combination index for *n* compounds (e.g., mycotoxins) at *x*% inhibition (e.g., proliferation inhibition); $(D_x)_{1-n}$ is the sum of the concentration of n compounds that exerts *x* % inhibition in combination, $\{[D_j]/\sum_1^n [D]\}$ is the proportionality of the concentration of each of n compounds that exerts *x*% inhibition in combination; and $(D_m)_j\{(fax)_j/[1-(fax)_j]\}^{1/mj}$ is the concentration of each compound alone that exerts *x*% inhibition. The CI < 1, =1, and >1 indicates synergism, additive and antagonism effect of the combination, respectively; The types of interactions produced by ZEA, α-ZOL and β-ZOL combinations were assessed by isobologram analysis using CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007).

2.5. Determination of ZEA, α-ZOL, β-ZOL and their metabolized products by LC–MS–LIT

For metabolic activity assays of the CHO-K1 cells on the mycotoxin ZEA and its metabolites (α-ZOL and β-ZOL), 10^4 cells per cm^2 were plated in 9 cm^2 polystyrene tissue culture dishes with culture medium and grown to confluence overnight. Fifty μM of each mycotoxins were added and uptake allowed to proceed for 24 and 48 h of exposure. For test control, ZEA and its metabolites were exposed to culture medium without CHO-K1 cells, during the same time of exposure.

The separation of ZEA and its metabolites was achieved by LC Agilent 1100 (Agilent Technologies, Santa Clara, California) coupled to a mass spectrometer (Applied Biosystems/MDS SCIEX Q TRAP TM linear ion trap mass spectrometer, Concord, Ontario, Canada). A Gemini (150 × 2.0 mm, 5 μm) Phenomenex (Torrance, California) column was used. LC conditions were set up using a constant flow at 0.3 ml/min, with water at 0.1% of formic acid (HCOOH) (phase A) and acetonitrile at 0.1% of HCOOH (phase B) as mobile phases in gradient condition were used. The gradient 9 parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350 °C, desolvation temperature 270 °C and collision gas energy 5 eV. The analyses of the ZEA, α-ZOL and β-ZOL were carried out employing the technique of the liquid chromatography coupled to the ion trap (LC–MS–LIT) with the following procedure: Characterization of the compound isolated with the modality of Enhanced Resolution scan, using the *m/z* range from 300 to 350 Da to obtain the general spectra of the degradation compound; The utilization of the mass spectrometer associated at the detection with the linear ion trap, utilized in this modality permitted us to obtain a good characterization of the compounds isolated (Juan et al., 2012). The method, for the detection and quantification of the mycotoxins targeted in this study was validated as a quantitative confirmatory method according to the EU Commission Decision, 2002/657/EC (Commission Decision, 2002) and the parameters taking into account for this purpose were: instrumental linearity, accuracy, precision (repeatability and reproducibility) and sensitivity. Linearity was evaluated using the standard calibration curves that were constructed for each mycotoxin by plotting the signal intensity versus the analyte concentration and the internal standard (I.S.) and obtaining the area ratios (area analyte/area internal standard). Calibration curves were constructed from

the peak area ratio of each analyte. The accuracy was evaluated through the calculation of individual compound recoveries. Recovery experiments were conducted at two different levels for each matrix, one at limits of quantification (LQs) and the other at 10 times LQs, added before the corresponding extraction procedure. Intra-day precision was assessed by calculating the relative standard deviation (RSDr), calculated from results generated under repetition conditions of six determinations per concentration in a single day. Inter-day precision was calculated by the relative standard deviation (RSDR) calculated from results generated under reproducibility conditions by one determination per concentration for 6 days. Sensitivity was evaluated by the limit of detection (LD) and LQ values.

2.6. Statistical analysis

Statistical analysis of data was carried out using SPSS statistic version 19.0 (SPSS, Chicago, IL, USA), statistical software package. Mean ± SD was used to express the results of four independent experiments. The statistical analysis of the results was performed by Student's *t*-test for paired samples. ANOVA followed by the Tukey HSD post hoc test for multiple comparisons were used to analyze statistically differences between groups. The level of $P \leqslant 0.05$ was considered statistically significant.

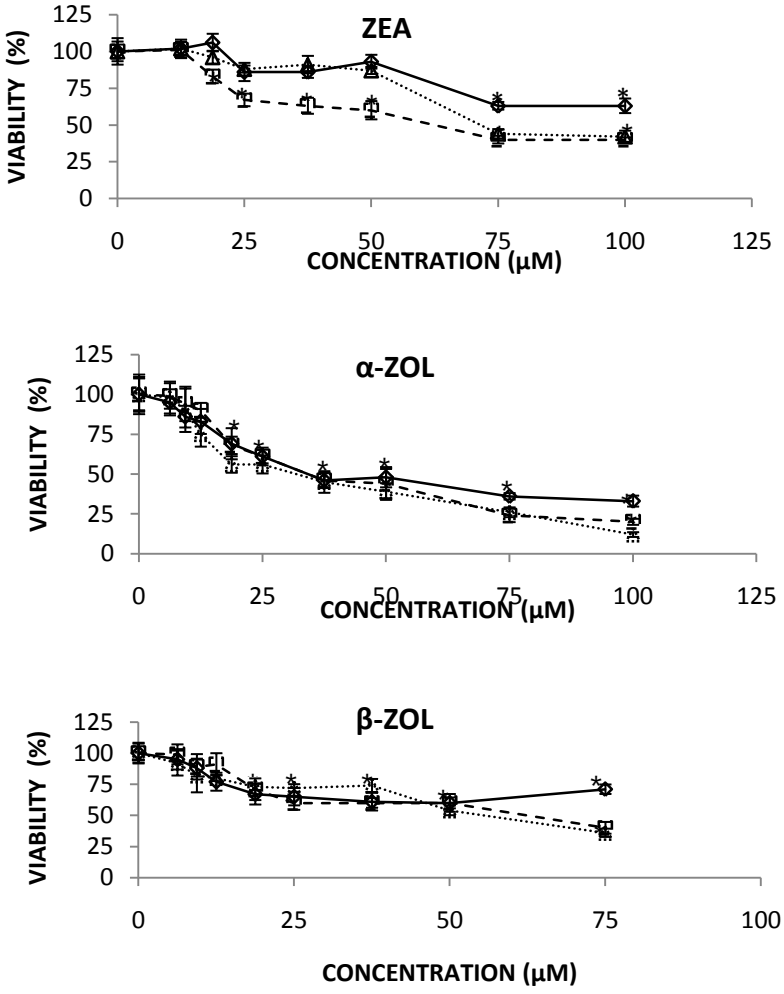
3. Results

3.1. Cytotoxicity assay of the individual and combined mycotoxins

The dose-response curves shown the IC_{50} values obtained by MTT method when the mycotoxins are alone after 24, 48 and 72 h of exposure

(Fig. 8). After 24 h of exposure, the IC_{50} values were >100, 33.00 ± 4.50 and >75 μM for ZEA, α-ZOL and β-ZOL, respectively. After 48 h of exposure, the IC_{50} values were 60.30 ± 5.99, 32.00 ± 5.15 and 62.50 ± 4.56 μM for ZEA, α-ZOL and β-ZOL, respectively. After 72 h of exposure, the IC_{50}values were 68.00 ± 2.55, 30.00 ± 5.50 and 55.00 ± 3.80 μM for ZEA, α-ZOL and β−ZOL, respectively. According to the IC_{50} values obtained the α-ZOL showed the highest cytotoxic effect on CHO-K1 cells.

Fig. 8. Dose-response curves for individual mycotoxins in CHO-K1 cells after 24 (◇), 48 (⊡) and 72 h (△) of exposure. Each point represents the mean value of at least three experiments. Data represents mean viability as mean ± SD (% of control). $p \leqslant 0.05$ (*) indicates a significant difference from control. The IC_{50} of ZEA, α-ZOL and β-ZOL was determined graphically from the concentration–response curves established for each mycotoxin.



The binary and tertiary mixtures of ZEA and its metabolites were studied using MTT assay in CHO-K1 cells after 24, 48 and 72 h of exposition. The dose-response curves of these three mycotoxin combinations are shown in Fig. 9 and Fig. 10, demonstrating a higher cytotoxicity comparing with individual test. Higher cytotoxicity observed with the binary and tertiary combinations is not surprising regarding the much higher concentration obtained after these mycotoxin combinations.

Fig. 9. Dose-response curves for ZEA (-◈-), α-ZOL (- -⊡- -), β-ZOL (⊙) and binary combinations (⋯△⋯) of them in CHO-K1 cells after 24, 48 and 72 h of exposure. Each point represents the mean value of at least three experiments. Data represents mean viability as mean ± SD (% of control). Concentration –response curves were fitted by non-linear regression. $p \leqslant 0.05$ (*) represent significant difference from control.

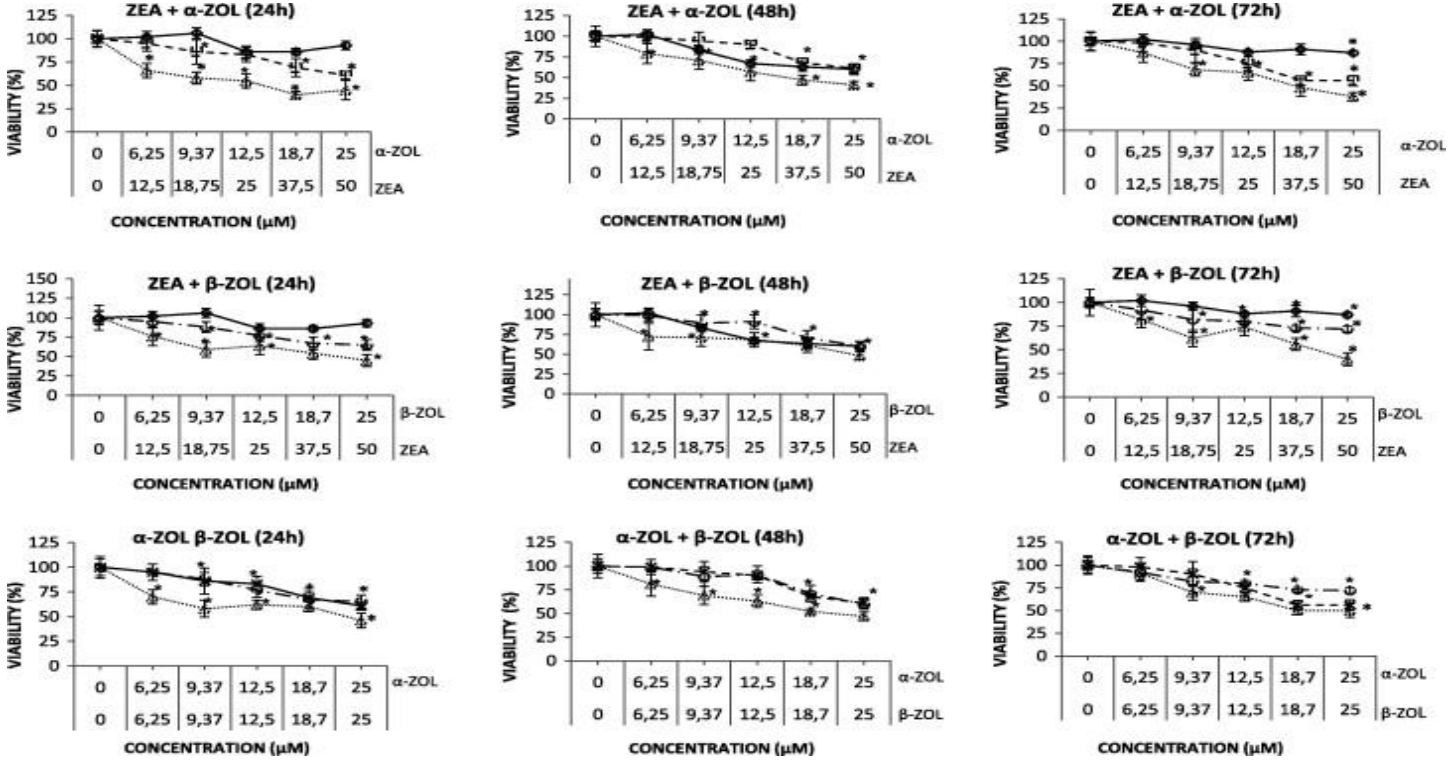
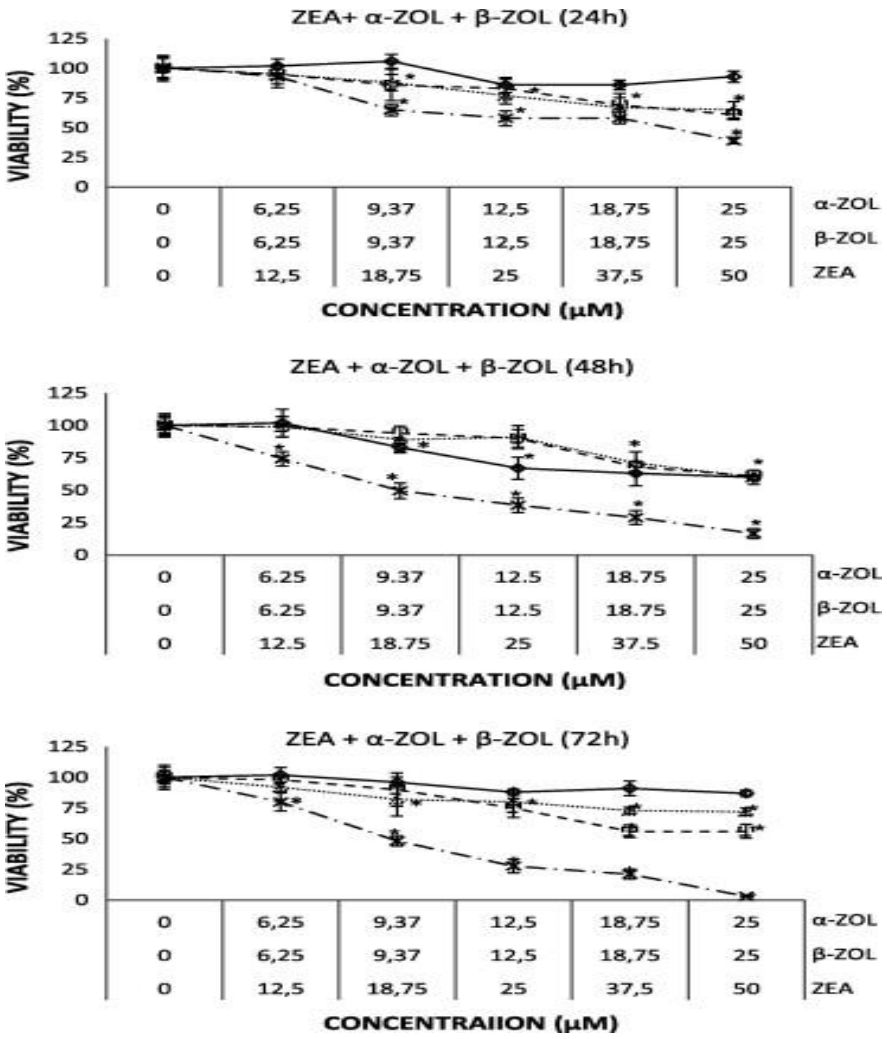


Fig. 10. Dose-response curves for ZEA (line and diamond), α-ZOL (dashed line and square), β-ZOL (···△···) and tertiary combinations (—✶—) of them in CHO-K1 cells after 24, 48 and 72 h of exposure. Each point represents the mean value of at least three experiments. Data represents mean viability as mean ± SD (% of control). Concentration-response curves were fitted by non-linear regression. $p \leqslant 0.05$ (*) represent significant difference from control.

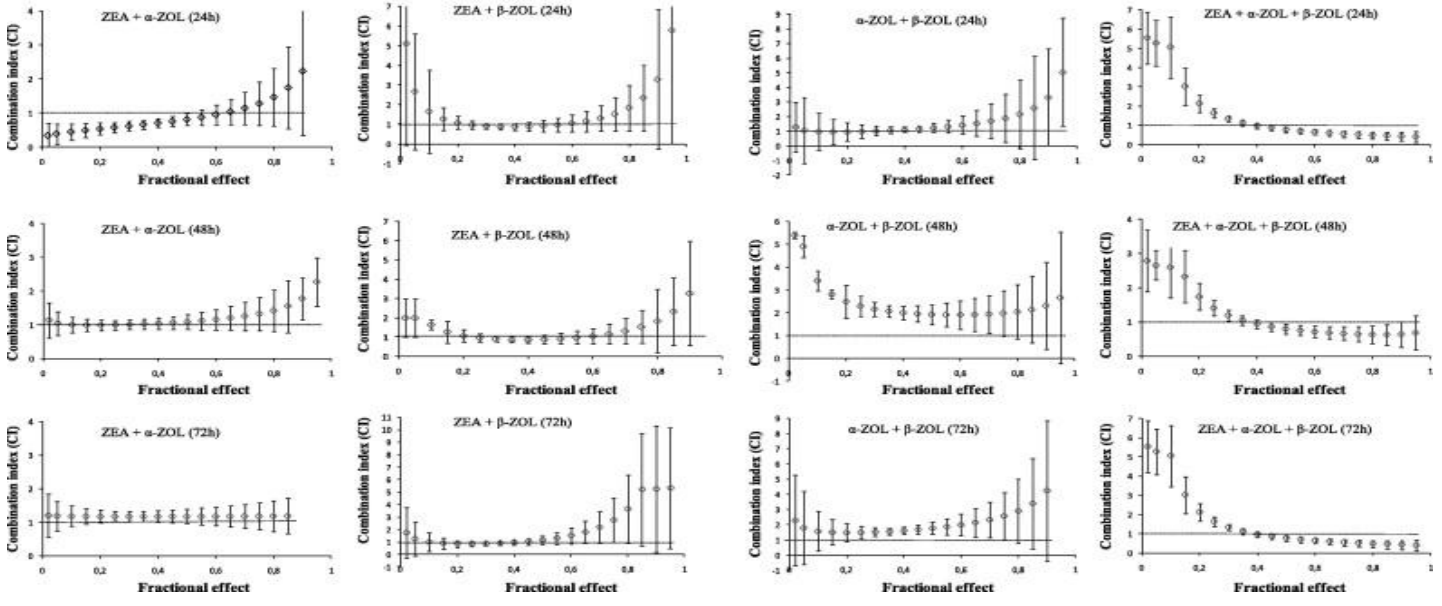


The isobologram analysis was used to determine the type of interaction between ZEA and its metabolites. The parameters D_m, m, and r of the binary and tertiary combinations, as well as mean combination index (CI) values are shown in Table 1. The CI_{25}, CI_{50}, $CI_{75}$and CI_{90} are the doses required to inhibit proliferation at 25%, 50%, 75% and 90%, respectively. These CI values were calculated automatically by the Computer software CalcuSyn. The CI/fractional effect (*fa*) curves for ZEA, α-ZOL and β-ZOL combinations in CHO-K1 cells are shown in Fig. 14. Fig. 14 demonstrates that a moderate synergism at high concentrations to the additive effects of low concentrations of ZEA + α-ZOL mixture after 24 h of exposure. However, after 48 and 72 h of exposure additive effect at all concentrations for ZEA + α-ZOL was demonstrated (Fig. 4; Table 11). Similarly, ZEA + β-ZOL mixture showed additive effects at all concentration and time of exposure (Table 11; Fig. 11). The mixture of α and β metabolites showed additives effect at 24 h, but at 48 and 72 h of exposure, the IC values showed antagonism at high concentration level and an additive effect at lower (Table 11; 11). The tertiary mixture showed an antagonism a higher concentrations and synergism a low concentrations (Table 11; Fig. 11).

Table 11. The parameters *m*, D_m and *r* are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose–effect curve, the potency (IC_{50}), and the conformity of the data to the mass-action law, respectively (Chou and Talalay, 1984 ; Chou, 2006). $D_m$and *m* values are used for calculating the CI value (CI < 1, =1, and > 1 indicates synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. $IC2_5$, IC_{50}, IC_{75} and IC_{90}, are the doses required to inhibit proliferation 25, 50, 75 and 90%, respectively. CalcuSyn Software provide automatically the IC_{25}, IC_{50}, IC_{75}, IC_{90} values.

MYCOTOXIN	TIME (h)	*Dm* (µM)	*m*	*r*	CI VALUES			
					CI_{25}	CI_{50}	CI_{75}	CI_{90}
ZEA	24h	153.02	1.06	0.9236				
	48h	52.33	1.65	0.9355				
	72h	88.53	2.05	0.9216				
α-ZOL	24h	43.30	1.05	0.9702				
	48h	37.79	1.58	0.9734				
	72h	28.45	1.29	0.9691				
β-ZOL	24h	88.45	0.55	0.9335				
	48h	33.93	5.85	0.9159				
	72h	74.59	0.82	0.9072				
ZEA + α-ZOL	24h	37.37	0.96	0.94693	0.56 ± 0.15 Syn	0.80± 0.18 Add	1.27± 0.63 Add	2.23± 1.90 Add
	48h	50.41	1.25	0.98778	0.99 ± 0.14 Add	1.09± 0.22 Add	1.32± 0.50 Add	1.77± 1.06 Add
	72h	48.05	1.76	0.93894	1.17 ± 0.15 Add	1.17± 0.21 Add	1.18± 0.40 Add	1.20± 0.63 Add
ZEA + β-ZOL	24h	57.97	0.88	0.97472	0.95 ± 0.20 Add	0.91± 0.27 Add	1.52± 0.86 Add	3.28± 2.71 Add
	48h	60.80	1.24	0.97471	0.90 ± 0.21 Add	1.09± 0.17 Add	1.43± 0.87 Add	3.02± 2.11 Add
	72h	72.27	0.68	0.95799	0.82 ± 0.20 Add	1.13± 0.36 Add	2.74± 1.81 Add	5.25± 5.10 Add
α-ZOL + β-ZOL	24h	35.96	0.64	0.89920	0.98 ± 0.20 Add	1.23± 0.30 Add	1.90± 1.65 Add	3.32± 3.33 Add
	48h	55.90	0.85	0.93920	2.28 ± 0.45 Ant	1.92 ± 0.43 Ant	1.97± 0.99 Add	2.30± 1.91 Add
	72h	51.47	0.67	0.92875	1.49 ± 0.41 Add	1.77 ± 0.40 Ant	2.57± 1.56 Add	4.24± 4.63 Add
ZEA + α-ZOL + β-ZOL	24h	55.33	4.80	0.89753	2.95 ± 0.75 Ant	1.50 ± 0.38 Ant	0.81 ± 0.33 Syn	0.54± 0.33 Syn
	48h	29.33	1.75	0.98144	1.41 ± 0.22 Ant	0.80 ± 0.14 Syn	0.64 ± 0.22 Syn	0.63± 0.36 Syn
	72h	27.94	2.54	0.98078	1.63 ± 0.24 Ant	0.50 ± 0.17 Syn	0.63 ± 0.36 Syn	0.41± 0.23 Syn

Fig. 11. Combination index (CI)/fractional effect curve as described by Chou and Talalay model in CHO-K1 cell exposed to ZEA, α-ZOL and β-ZOL binary and tertiary combiantions. Each point represents the CI ± SD at a fractional effect as determined in our experiments. The dotted line (CI = 1) indicates additivity, the area under the dotted line synergy, and the area above of the dotted line antagonism. CHO-K1 cells were exposed during 24, 48 and 72 h with ZEA + α-ZOL and ZEA + β-ZOL at molar ratio of 2:1; α-ZOL + β-ZOL at molar ratio of 1:1 (equimolar proportion) and ZEA + α-ZOL + β-ZOL at molar ratio of 2:1:1.



According to the CI_{50}, the cytotoxic effect of the combinations decreased in the following order: (a) after 24 h of exposure ZEA + α-ZOL < ZEA + β-ZOL = α-ZOL + β-ZOL = ZEA + α-ZOL + β-ZOL and (b) after 48 and 72 h of exposure, ZEA + α-ZOL + β-ZOL < ZEA + α-ZOL = ZEA + β-ZOL < α-ZOL + β-ZOL (Table 11; Fig. 11).

3.2. Metabolization of ZEA, α-ZOL and β-ZOL by CHO-K1 cells

The linearity in LC–MS/MS was obtained in triplicate by spiking six concentrations (10, 20, 50, 100, 250 and 500 μM). The linear regression coefficient of calibration curves showed a correlation coefficient (r^2) higher than 0.992. Recovery experiments were conducted at two different levels for each matrix, one between 5 and 50 μM (LQs) and the other between 50 and 500 μM (10 times LQs). In both cases, mycotoxins added before the corresponding extraction procedure evidenced a recovery ranged from 75% to 99%. The values of intra-day precision (n = 6) and inter-day precision (n = 6) were inferior to 10% and 14%, respectively.

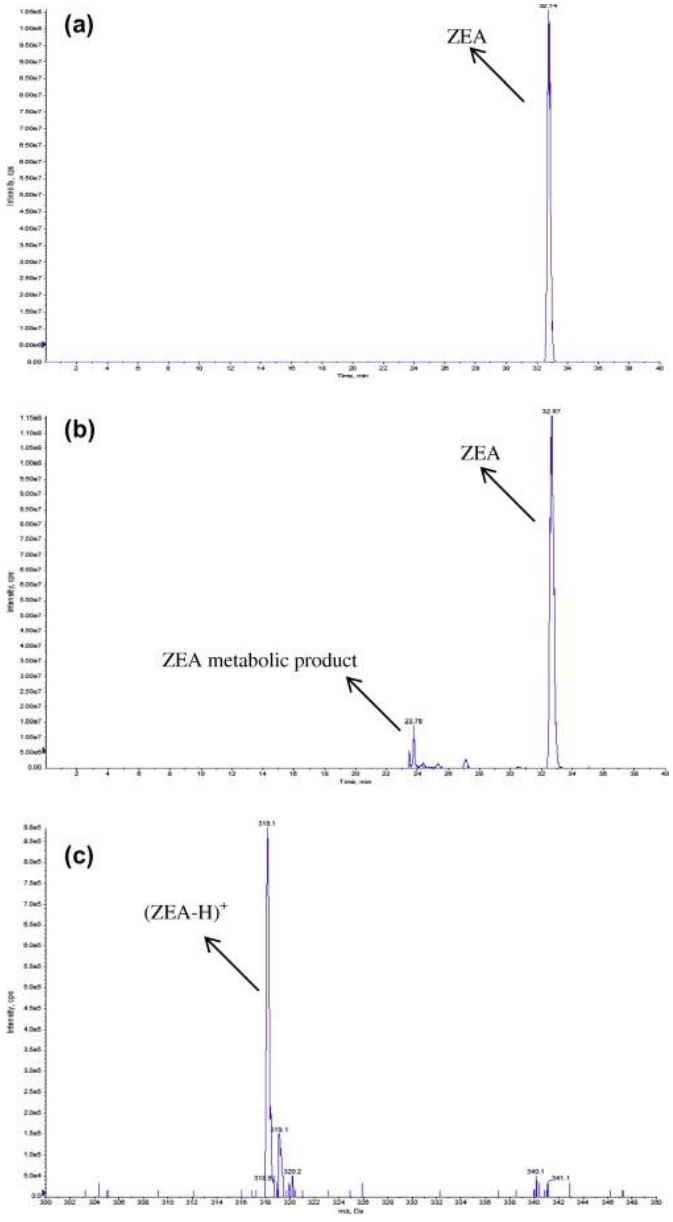
Sensitivity was evaluated by LD and LQ values. The LD was estimated from blank extract, spiked with decreasing concentrations of the analytes. The response of the mycotoxin peak was equal to 3 times the response of the blank extract (n = 20). Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LD and LQ evidenced for the mycotoxins studied ranged from 1 to 5 μM.

To determine the metabolic capacity of CHO-K1 cells transforming the ZEA into α-ZOL and β-ZOL, the CHO-K1 cells were exposed to 50 μM of each mycotoxins during 24 and 48 h of incubation. The growth medium exposed to mycotoxins without cells was considered as control. The metabolic change evidenced by CHO-K1 cells on the mycotoxin ZEA, α-

ZOL and β-ZOL at 24 h was 4.0 ± 0.9, 8.6 ± 1.1 and 10.4 ± 2.2%, respectively. At 48 h incubation, it is possible to observe an increase of the metabolic activity promoted by CHO-K1 cells on the mycotoxins studied. The degradation evidenced on ZEA, α-ZOL and β-ZOL was 81.3 ± 3.1, 79.1 ± 2.2 and 85.0 ± 3.6, respectively.

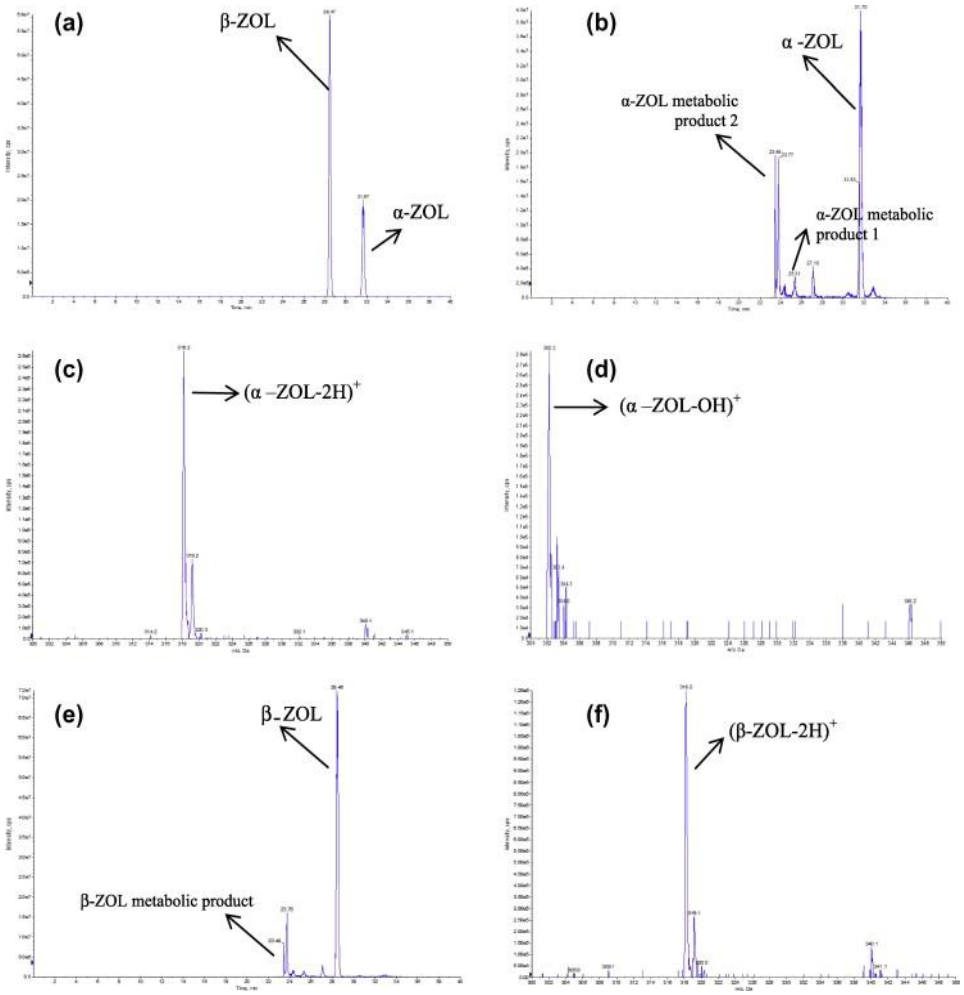
To detect the metabolic change of ZEA, α-ZOL and β-ZOL, the growth medium exposed these mycotoxins was collected and injected in the LC–MS–LIT. Fig. 5a shows the LC–MS–LIT chromatogram of the ZEA (retention time, RT, 32.74 min) present in the control solution (medium without cells). The LC–MS–LIT chromatogram in 12b evidences the ZEA (50 μM) present in the growth medium and one of its products metabolized by CHO-K1 cells, which was identified as the ZEA without a hydrogen group.

Fig. 12. LC–MS–LIT chromatogram of (a) ZEA control samples (ZEA present in the growth medium without CHO-K1 cells), (b) ZEA metabolized by CHO-K1 cells and products generated and (c) LC–MS–LIT spectrum of ZEA metabolites produced in CHO-K1 cells.



The LC–MS–LIT chromatogram in Fig. 6a shows the α-ZOL and β-ZOL which RT are 28.4 and 31.6 min, respectively. The Fig. 13b shows the LC–MS–LIT chromatogram of the α-ZOL where are evidenced also two other degradation peaks related to two metabolic products of the α-ZOL. The first one (Fig. 13c) was identified as the α-ZOL with the loss of two hydrogen groups by the diagnostic fragment with *m/z* of 318.2. The second α-ZOL metabolic product produced by CHO-K1 cells (Fig. 13d) showed the loss of a hydroxil group as evidenced by *m/z* of 302.3. In Fig. 6e and Fig. 6f are shown the LC–MS–LIT chromatograms related to the β-ZOL metabolized by the CHO-K1 cells. There is a metabolic product characterized as β-ZOL with the loss of two hydrogen groups as evidenced by the fragment with a *m/z* of 318.2. Comparing the RT of α-ZOL and β-ZOL (Fig. 13a) with the peaks present in the chromatogram evidenced in the Fig. 13b is possible to deduce that the ZEA in CHO-K1 cells is not metabolized into α-ZOL and β-ZOL. But it was demonstrated that these mycotoxin can produce other degradation products.

Fig. 13. LC–MS–LIT chromatogram of the (a) α-ZOL and β-ZOL control samples (α-ZOL and β-ZOL present in the growth medium without CHO-K1 cells), (b) metabolites generated by α-ZOL in CHO-K1 cells, (c) LC–MS–LIT spectrum of α-ZOL metabolite (product 1) produced in CHO-K1 cells, (d) LC–MS–LIT spectrum of α-ZOL metabolite (product 2) produced in CHO-K1 cells, (e)metabolite generated by β-ZOL in CHO-K1 cells and (f) LC–MS–LIT spectrum of β-ZOL metabolite produced in CHO-K1 cells.



4. Discussion

The cytotoxicity produced by ZEA has been widely studied using different cell cultures and different endpoints of determination. CHO-K1 cells were selected because previous works demonstrated that ZEA and its metabolites are distributed to estrogen target tissues/cells as ovarian. Data regarding the two major metabolites (α-ZOL and β-ZOL) are scarce. Still the results obtained from them differ respect to which of the two mycotoxins (α-ZOL or β-ZOL) is the most toxic. Abid-Essefi et al. (2009) and Ayed et al. (2011) demonstrated by MTT method, that ZEA was more cytotoxic than α-ZOL and β-ZOL when exposed during 24 (Caco-2 cells) and 48 h (HeLa cells) at different concentrations of these mycotoxins. Instead, Marin et al. (2010) determined that the ZEA was less cytotoxic than α-ZOL and β-ZOL when neutrophils were exposed to these mycotoxins. There are also differences respect from which metabolite is more cytotoxic. Abid-Essefi et al. (2009) and Othmen et al. (2008) obtained the metabolite β-ZOL is more cytotoxic in Caco-2 and Vero cells than α-ZOL. However, Ayed et al. (2011) found similar results to those obtained in this study. They found that the α-ZOL is the more cytotoxic metabolite in Hela and Vero cells after 24 h of exposure than β-ZOL.

The results obtained in this study to come to the conclusion that high concentration of ZEA and its metabolites are required to achieve the IC_{50} values in the CHO-K1 cells. Similarly the *in vivo* studies have indicated that ZEA has low acute toxicity (Kuiper-Goodman et al., 1987).

Occurrence of mycotoxins in food commodities is usual. So, it is important to know whether interaction between mycotoxins may increase or decrease their toxic effect on the consumer. The ZEA is rapidly metabolized in various tissues, particularly in the liver to its two major metabolites (α-ZOL and β-ZOL). And mixtures of these mycotoxins may be

present in biological systems and to be a health hazard depending on the type of interaction that takes place.

In the ZEA + α-ZOL combination (Fig. 2), at 24 and 72 h of exposure, there was a decrease in cell proliferation at the highest concentration: approx. 66% of ZEA tested in combination against ZEA tested individually, and almost 25% for α-ZOL. After 48 h of exposure, there was a smaller decrease (about 10%) for the mixture respect both, ZEA and α-ZOL, individually. After 24 h of exposure, ZEA + β-ZOL combination (Fig. 2) decreased cell proliferation about 50% and 10% at the highest concentration against ZEA and β-ZOL tested individually, respectively. After 48 h of exposure, the highest concentration of the combination reduced cell proliferation approx. 10% compared these mycotoxins tested individually. And, at 72 h of exposure, the combination decreases approximately 65% and 25% with respect to ZEA and β-ZOL individually tested. As can be observed in Fig. 2, α-ZOL + β-ZOL combination showed at all times of exposure a similar decreased in cell proliferation. Decreased in cell proliferation of the mixture at the highest concentration tested was almost 20% compared to α-ZOL and β-ZOL tested alone. Fig. 3 shows the dose-response curves for the tertiary combination of ZEA and its metabolites at 24, 48 and 72 h of exposure in CHO-K1 cells. At 24 h of exposure, cell proliferation decreased approximately 25% when cells were exposed to the tertiary combination compared to the metabolites tested individually and 50% against ZEA individually tested. After 48 h of exposure, a similar reduction in cell proliferation (45%) was observed for ZEA + α-ZOL + β-ZOL compared with each mycotoxin individually assayed. The greatest decrease in viability was observed at 72 h of exposure, where the cell proliferation of the combination tertiary decreased in 50%, 70%, 80% compared to the viability of α-ZOL, β-ZOL and ZEA alone, respectively. All combinations

showed greater cell viability reduction than mycotoxins tested alone, due in part to a higher concentration of mycotoxins in the culture medium. Moreover, reduction of CHO-K1 proliferation was higher for ZEA than metabolites, which means that metabolites play an important role in the toxic effect of the mixtures.

Kouadio et al. (2007) studied the cytotoxicity that occurs when Caco-2 cells were exposed to DON, FB_1 and ZEA. They demonstrated that ZEA + FB_1 and ZEA + DON mixtures produced an additive effect and the tertiary combination produced synergism. Bouaziz et al., 2012 showed that ZEA + T-2 toxin produced synergism after 24 h of exposure on Vero cells. Similarly, Luongo *et al*., 2006 ; Luongo *et al*., 2008 observed that α-ZOL + FB1 produced synergism on Jurkat and porcine whole-blood cells. Consequently, the interactions produced by the mycotoxins when they are in combination are difficult to explain. As could be observed, the effects that produce mixtures of mycotoxins differ in the type of cells exposed, mixtures of mycotoxins, the ratio used for each mycotoxin in the mixture, the method used to determine the dose effect relationship existing and exposure time. Moreover, metabolic processes are very complicated and involving multiple factors; the affinity of ZEA and its metabolites by the ER depends of different functional groups and the spatial distribution to have a lower or higher affinity for each receptor. And, the exact mechanism of action that produces ZEA and its metabolites does not completely understood.

In toxicological studies, the fact that extracellular metabolites somehow reflect the intracellular situation is of major importance. The *in vitro* ZEA, α-ZOL and β-ZOL metabolization approach could be proven advantageous in mycotoxins toxicity. A toxicological screening of degradation of these mycotoxins in a mammalian cell culture provides insights into their potential toxic effects in a target organ/system *in vivo*. In that way, in the regulatory

context could be discussing the current status related to the safety assessment of ZEA and its metabolites. The ZEA can be metabolizing in α-ZOL and β-ZOL by enzymatic components of cell lines has been previously evaluated. Miles et al. (1996) evaluated ZEA metabolism in its ZOL compounds by an *in vivo* sheep model. They demonstrated that sheep degrade the mycotoxin studied with a metabolic patter consistent with the ovine metabolism. Videmann et al. (2008) studied the metabolism and the transfer of ZEA in Caco-2 cells as a model of intestinal epithelium. The cells were exposed to ZEA (10–100 μM) evidencing an efficacious metabolism of the ZEA into α-ZOL and β-ZOL, which were the more abundant metabolites. In particular, the 40.7% of the mycotoxin ZEA was converted into α-ZOL, and 31.9% in β-ZOL. Also, other glycosylated ZEA metabolites were identified. Dong et al. (2010) evaluated the conversion of the ZEA into α-ZOL and β-ZOL by liver subcellular fractions. They observed that in the liver, α-ZOL was a major metabolite in cytosol and β-ZOL in microsome fractions. Then, mitochondrial fractions of the liver converted ZEA predominantly to α-ZOL, indicating that the goat liver may function as an activation organ rather than as an inactivation organ, for ZEA metabolism in goats. In other tissues including rumen tissue, the ZEA metabolism into α-ZOL was higher than β-ZOL.

Results obtained in this study, shown that CHO-K1 cells can be used for the identification of ZEA metabolites in *in vitro* system. The metabolites generated may be specific for this type of cells, but some may allow general predictions from *in vitro* to *in vivo*. However, further investigation must focus on the toxicity of the products generated by the metabolism between CHO-K1 and the mycotoxins studied. The characterization of the toxicological parameters of these newly discovered substances is of

paramount importance to complete the toxicity of all the compounds related to ZEA family.

In summary, the data evidenced in this study showed that α-ZOL was more cytotoxic than ZEA and β-ZOL to CHO-K1 cells. Binary and tertiary mycotoxin combinations, related to decrease cell viability in CHO-K1 cells showed a higher cytotoxic effect than mycotoxins tested alone. Decreasing cell viability was dose and time of exposure dependent. Fifty μM of ZEA, α-ZOL and β-ZOL were metabolized into dehydrogenation and dehydroxilation products at 24 and 48 h of exposure. No conversion of ZEA in α-ZOL and β-ZOL was produced by CHO-K1 cells. Results obtained *in vitro* in mammalian CHO-K1 cells will be provided qualitative and quantitative information about ZEA, α-ZOL and β-ZOL *in vivo* risk assessment predictions.

Evaluation of the toxicity of ZEA and its metabolites in humans, considering all relevant toxicological endpoints and identification of the different metabolites produced in biological systems of the toxicological relevance present in food is an issue of health concern. Moreover, ZEA co-occurs with other *Fusarium* mycotoxins such as deoxynivalenol, nivalenol and fumonisins. So, the exposure to these mycotoxins should be reduced to As Low As Reasonably Achievable (ALARA). However, there are gaps in the toxicological information of these mycotoxins, including exposure assessment, metabolites generated, characterization of adverse effects of ZEA and its metabolites, and the toxicological assessment of the co-occurrence of these mycotoxins. Concluding, it will be important to identify ZEA in feed commodities and to consider the rise of combining multi-mycotoxins metabolites and the extent of their charge to predict toxicity. Additional comparative studies on species differences (including use of

human cells) in the metabolism of ZEA to elucidate the differential findings in long term toxicity are needed.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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3.2 Cytotoxic and interactive effect of Zearalenone, α-zearalenol and β-zearalenol and formation of metabolites in HepG2 cells

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Cytotoxic and interactive effect of Zearalenone, α-zearalenol and β-zearalenol and formation of metabolites in HepG2 cells

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Abstract

Zearalenone (ZEA) is a secondary metabolite of *Fusarium* fungi. ZEA is a non-steroidal estrogenic mycotoxin which is rapidly absorbed and metabolized to α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) in the liver; therefore mixtures of these mycotoxins may be simultaneously present in biological systems and cause human health risk. The objectives of this study were: a) to compare the cytotoxicity of ZEA, α-ZOL and β-ZOL alone or in combination on human hepatoma (HepG2) cells using the MTT assay after 24, 48 and 72h of exposure, and b) to evaluate the interactions of these mycotoxins mixtures in HepG2 cell lines by the isobologram analysis. The IC_{50} values obtained for individual mycotoxins range from 70.0 to >100.0 μM, from 20.6 to 26.0 μM and from 38.4 to >100.0 μM in HepG2 cells for ZEA, α-ZOL and β-ZOL, respectively. Isobologram analysis provides a combination index (CI) value to determine the type of interaction that occurs. The interactions of ZEA and its metabolites showed slightly synergism (CI from 0.34 ± 0.10 to 0.69 ± 0.22) followed by additive effect (CI from 0.97 ± 0.20 to 2.61 ± 2.15) and turned into antagonism (CI from 1.29 ± 0.18 to 7.77 ± 2.27). The concentration of ZEA and its metabolites was determined with liquid chromatography coupled to the mass spectrometer detector-linear ion trap (LC-MS-LIT). No conversion of ZEA in

α-ZOL and β-ZOL was detected. Howeverother degradation products were detected.

Keywords: zearalenone, HepG2 cells, interactive effect, isobolas, metabolites

Citotoxicidad, formación de metabolitos e interacción entre Zearalenona, α- zearalenol y β-zearalenol en células HepG2

La zearalenona (ZEA) es un metabolito secundario producido por hongos del género *Fusarium*. ZEA es una micotoxina estrogénica no esteroidea que se metaboliza rápidamente en el hígado a α-zearalenol (α-ZOL) y β-zearalenol (β-ZOL); por lo tanto, mezclas de estas micotoxinas pueden estar presentes simultáneamente en un sistema biológico y causar un riesgo para la salud en el hombre. Los objetivos de este estudio fueron: a) comparar la citotoxicidad de la ZEA, α-ZOL y β-ZOL de forma individual y en combinación en células hepáticas humanas (HepG2) usando el ensayo MTT tras una exposición de 24, 48 y 72h y b) evaluar la interacción de las mezclas de estas micotoxinas en las células HepG2 mediante el análisis de las isobolas. Los valores de IC50 obtenidos en células HepG2 con las micotoxinas individuales van desde 70,0 a > 100,0 μM, de 20,6 a 26,0 μM y de 38,4 a > 100,0 μM para ZEA, α-ZOL y β-ZOL, respectivamente. El método de las isobolas proporciona el índice de combinación (IC) con el que se determina el tipo de interacción que se produce entre las micotoxinas. La interacción entre la ZEA y sus metabolitos mostró un ligero sinergismo (CI de 0,34 ± 0,10 a 0,69 ± 0,22), seguido de efecto aditivo (CI de 0,97 ± 0,20 a 2,61 ± 2,15) que se acabó en antagonismo (CI de 1,29 ± 0,18 a 7,77 ± 2,27) dependiendo de la concentración y tiempo de exposición. La concentración de ZEA y sus metabolitos se determinó con cromatografía líquida acoplada a espectrómetría de masas con trampa de

iones lineal (LC-MS-LIT). No se detectó ninguna conversión de ZEA en α-ZOL y β-ZOL. Sin embargo se detectaron otros productos de degradación.
Palabras clave: Zearalenona, células HepG2, interacción, isobolas, metabolitos

1 Introduction

Mycotoxins are secondary metabolites of fungi. There are more than 300 mycotoxins and their effects have not been completely characterized [1]. Zearalenone (ZEA) is a mycotoxin produced as a secondary metabolite of *Fusarium* fungi [2-3]. ZEA is metabolized in the liver in α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) by 3α-hydroxisteroid dehydrogenase and 3β-hydroxisteroid dehydrogenase, respectively [4]. ZEA biotransformation also can occur in fungi and plants, which are involved in the aromatic ring and aliphatic group [3].

ZEA is hepatotoxic which can develop into hepatocarcinoma. Besides, ZEA is hematotoxic, inmunotoxic and genotoxic [5-6]. However, reproductive system is the target organ of ZEA and its metabolites. They produced endocrine disruption which causes estrogenic effects. These alterations are due to the ability of ZEA and its metabolites of binding to the estrogenic receptors (ER) [4,7,8].

The Food and Agriculture Organization (FAO) estimates that approximately 25% of world production of food is contaminated with at least one mycotoxin [1, 9]. Human and animals are exposed simultaneously to several *Fusarium* mycotoxins for different reasons: 1-*Fusarium* fungi are able to produce more than one mycotoxin; 2- food and feed can be contaminated by several fungi simultaneously and 3- a complete diet is made up of different commodities [10]. The natural co-occurrence of mycotoxins in food and feed is a cause of concern because the exposure to

mixture of mycotoxins may be potent and cause more damage to human health than mycotoxins alone [10, 11, 12]. However, the ability of ZEA to metabolize into α-ZOL and β-ZOL can produce mixtures of these mycotoxins in the organism which may cause an increase in the toxicity produced by these mycotoxins. On the other hand, the interactive effect of the mycotoxins is poorly understood and the data of these mixtures are limited, besides the health problems of the exposure to the combination mycotoxins are unknown. So, to study the interaction between mycotoxins is needed; besides it is known that *Fusarium* mycotoxins can produces synergism and additive [13, 14, 15, 16, 17] effects.

The cellular cytotoxicity assays have been increasing since they are faster to apply, cheaper, easier to maintain, and they are more quantitative methods than *in vivo* assay. Moreover, the experimental conditions can be controlled easily, they are widely reproducible and avoid the ethical problems associated with in vivo experimentation [18]. There are a lot of studies about the cytotoxicity of ZEA tested alone, but only a few studies about combined effects of ZEA or its metabolites [1, 5]. And only few of these authors used the Isobologram method [14, 17].

Due to ZEA is metabolized by the liver into α-ZOL and β-ZOL, the aim of study was to determinate the cytotoxic effects of ZEA and its metabolites tested alone and in combination in HepG2 cells after 24, 48 and 72h of incubation. And the type of interaction of these mycotoxins in the mixtures. The HepG2 cells which posse metabolic capacity were selected to prove if this cell line can metabolize ZEA in its two metabolites or even other different.

2 Materials and methods

2.1 Reagents and equipment

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, 3,4,5-dimethylthiazol-2-yl, 2,5-diphenyltetrazolium bromide (MTT), phosphate buffer saline (PBS), glucose, dimethyl sulfoxide (DMSO), and analytical standard of mycotoxins were from Sigma Chemical Co. (St Louis, MO, USA). Mycotoxins selected were ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionized water (resistivity <18 MX cm) was obtained using a purification system Milli-Q (Millipore, Bedford, MA, USA). All other reagents were of standard laboratory grade. Stock solutions of mycotoxins were prepared in methanol and maintained at -20°C in the dark. The final concentrations tested were achieved by adding the culture medium with mycotoxins and the final methanol concentration in medium was 1% (v/v).

For analytical assay, a LC coupled to a mass spectrometer (Applied Biosystems/MDS SCIEX Q TRAP TM linear ion trap mass spectrometer, Concord, Ontario, Canada) with A Gemini (150 x 2.0 mm, 5 μm) Phenomenex (Torrance, California) column was used.

2.2. Cell culture and treatment

Cells were grown at 37 °C in 9 cm^2 polystyrene tissue culture dishes. HepG2 cells were grown using DMEM medium. Media were supplemented with 25mM HEPES buffer (pH 7.4), 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin. The incubation conditions were 5% CO_2 at 37ºC and 95% air atmosphere at constant humidity. Absence of mycoplasma was

checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

2.3. Cytotoxicity of mycotoxins

Cytotoxicity assays were determined by the MTT assay performed as described by Ruiz [19].

Mammalian HepG2 cells were cultured in 96-well microplates by adding 200 µL/well of a suspension of 2 x 10^4 cells/well. After cells reached 90% confluence, the culture medium was removed and was added fresh medium containing the serial dilution of each mycotoxin: ZEA (from 12.5 to 100 µM), α-ZOL (from 6.5 to 100 µM) and β-ZOL (from 6.5 to 100 µM) incubated during 24, 48 and 72 h. Then, the medium was removed and cells of each well received fresh medium containing 50 µl MTT. After 4 h of incubation at 37ºC under darkness, the resulting formazan was solubilized in DMSO. The absorbance was measured at 570 nm using an automatic ELISA plate reader (MultisKanEX, Labsystem, Helsinki, Finland). The IC_{50} values were obtained from full concentration response curve.

To carry out the cytotocity assay with the mixtures, HepG2 cells were incubated with several dilutions of each binary and tertiary combinations of ZEA, α-ZOL and β-ZOL. Mixtures were prepared as follow: ZEA + α-ZOL and ZEA + β-ZOL at constant 2:1 ratio, α-ZOL + β-ZOL at constant 1:1 ratio and ZEA + α-ZOL + β-ZOL at constant 2:1:1 ratio. Five dilutions of each mycotoxin combinations (ZEA 12.5-100; α-ZOL 6.25-100 and β-ZOL 6.25-100) plus a control were tested in three independent experiments.

2.4 Interactions between mycotoxins

The median-effect/combination index (CI)-isobologram equation by Chou [20] and Chou and Talalay [21] was used to analyzed the results

which is based on the median-effect principle (mass-action law) that demonstrates that there is an univocal relationship between dose and effect independently of the number of substrates or products and of the mechanism of action or inhibition. The CI index was calculated by the equation:

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

Where $^{n}(CI)_x$ is the combination index for n compounds (e.g., mycotoxins) at *x*% inhibition (e.g., proliferation inhibition); $(D_x)_{1-n}$ is the sum of the concentration of n compounds that exerts *x*% inhibition in combination, $\{[D_j]/ \sum^{n}_{1} [D]\}$ is the proportionality of the concentration of each of n compounds that exerts *x*% inhibition in combination; and $(D_m)_j \{(fax)_j/[1-(fax)_j]\}^{1/mj}$ is the concentration of each compound alone that exerts *x*% inhibition. The result from this equation, CI < 1, = 1, > 1 indicates synergism, additive or antagonism effect, respectively. The types of interaction shaped by the combinations of ZEA and its metabolites were assessed by isobologram analysis using CalCusyn software (Biosoft, Cambrige, UK)

2.5 Determination of ZEA, α-ZOL, β-ZOL and their degradation products by LC-MS-LIT

The determination of ZEA and its metabolites on HepG2 cells was carried out using a liquid chromatography coupled to linear ion trap mass

spectrometry (LC/MS/LIT). Ten thousand cells per cm^2 were plated in 9 cm^2 polystyrene tissue culture dishes with culture medium and grown to confluence overnight. Fifty µM of ZEA, α-ZOL and β-ZOL were added and incubated for 24 and 48 h. After, the medium and cells were collected extracted and analyzed by LC/MS/LIT. Cells were collected in an eppendorf, 1 µl of growth medium was added and ultrasonicated during 3h for breaking cells. After that, an extraction of mycotoxins with ethylacetate was made and the extract was dried in turbobap and finally redissolved with 1ml of methanol. Respect to the growth medium the same extraction procesure was performed for mycotoxins.

LC conditions were set up using a constant flow at 0.3 mL/min, with water at 0.1% of formic acid (HCOOH) (phase A) and acetonitrile at 0.1% of HCOOH (phase B) as mobile phases in gradient condition were used. The gradient employed was: 0-5 min-5% B, 15-25 min-90% B, 35-40 min-5% B. The instrument was configured in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350ºC, desolvation temperature 270ºC and collision gas energy 5 eV. The analyses of the ZEA, α-ZOL and β-ZOL were carried out employing the technique of LC-MS-LIT with the following procedure: Characterization of the compound isolated with the modality of enhanced resolution scan, using the *m/z* range from 300 to 350 Da to obtain the general spectra of the degradation compound; The utilization of the mass spectrometer associated at the detection with the LIT, utilized in this modality permitted us to obtain a good characterization of the compounds isolated [22]. The method, for the detection and quantification of the mycotoxins targeted in this study was validated as a quantitative confirmatory method according to the EU Commission Decision, 2002/657/EC [23] and the parameters taking into account for this purpose

were: instrumental linearity, accuracy, precision (repeatability and reproducibility) and sensitivity. Linearity was evaluated using the standard calibration curves that were constructed for each mycotoxin by plotting the signal intensity versus the analyte concentration and the internal standard (I.S.) and obtaining the area ratios (area analyte/area internal standard). Calibration curves were constructed from the peak area ratio of each analyte. The accuracy was evaluated through the calculation of individual compound recoveries. Recovery experiments were conducted at two different levels for each matrix, one at limits of quantification (LQs) and the other at 10 times LQs, added before the corresponding extraction procedure. Intra-day precision was assessed by calculating the relative standard deviation (RSDr), calculated from results generated under repetition conditions of six determinations per concentration in a single day. Inter-day precision was calculated by the relative standard deviation (RSDR) calculated from results generated under reproducibility conditions by one determination per concentration for 6 days. Sensitivity was evaluated by the limit of detection (LD) and LQ values.

3 Results

3.1 Cytotoxicity produced by individual mycotoxins

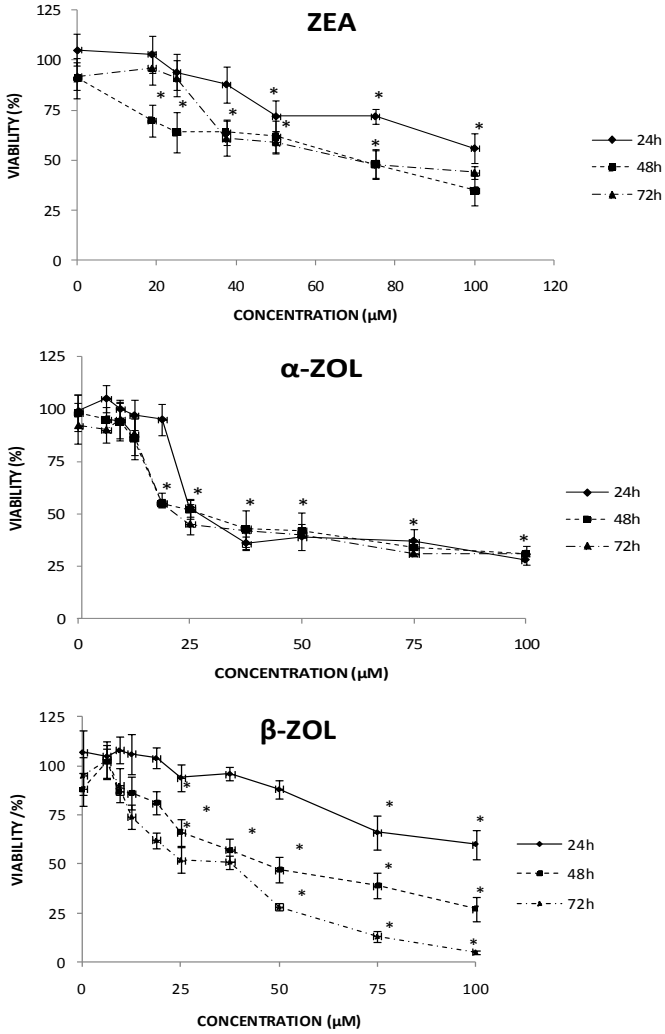
The concentration-response curves of individual mycotoxins in HepG2 cells are shown in Figure 14. It can be observed that ZEA and metabolites inhibited cell growth in dose and time dependent manner.

The IC_{50} values, observed in Table 12, demonstrate that at 24h α-ZOL showed the lowest IC_{50} value, whilst is maintained over time. ZEA and β-ZOL showed similar IC_{50} values at 24h. But, at 48 and 72h β-ZOL shows lower IC_{50} than ZEA.

Table 12. IC_{50} values of ZEA, α-ZOL and β-ZOL obtained on HepG2 cells, after 24, 48 and 72h of exposure.

Mycotoxin	IC_{50} (μM)		
	time (h)		
	24	48	72
ZEA	>100	71.30 ± 5.70	70.00 ± 5.70
α-ZOL	27.00 ± 4.00	25.50 ± 4.20	20.60 ± 4.50
β-ZOL	>100	45.00 ± 5.56	38.40 ± 3.47

Figure 14. Concentration-response curves for individual mycotoxins on HepG2 cells after 24(—◆—), 48 (---■---) and 72 h (····▲····) of exposure. Each point represents the mean value of at least three experiments. Data are expressed as mean value ± SD (% of control). $p \leq 0.05$ (*) indicates a significant difference from control.



3.2 Cytotoxicity and interaction produced by mycotoxins combination

Figure 15 shows the concentration-response curves of mixtures of mycotoxins in the HepG2 cells at 24, 48 and 72 h of exposure.

In the combination of ZEA + α-ZOL (Fig. 15), at 24h of exposure, there was a decrease in cell viability of approximately 20% at the highest concentration tested in combination, against ZEA individually tested. After 48 h of exposure, there was a decrease of approximately 10% and 20% at the highest concentrations tested in combinations, against α-ZOL and ZEA tested alone respectively. While, after 72h of exposure, the cell viability was reduced approximately 40% for this combination when compared with individual ZEA, and the reduction of the viability was 30% respect to α-ZOL tested alone.

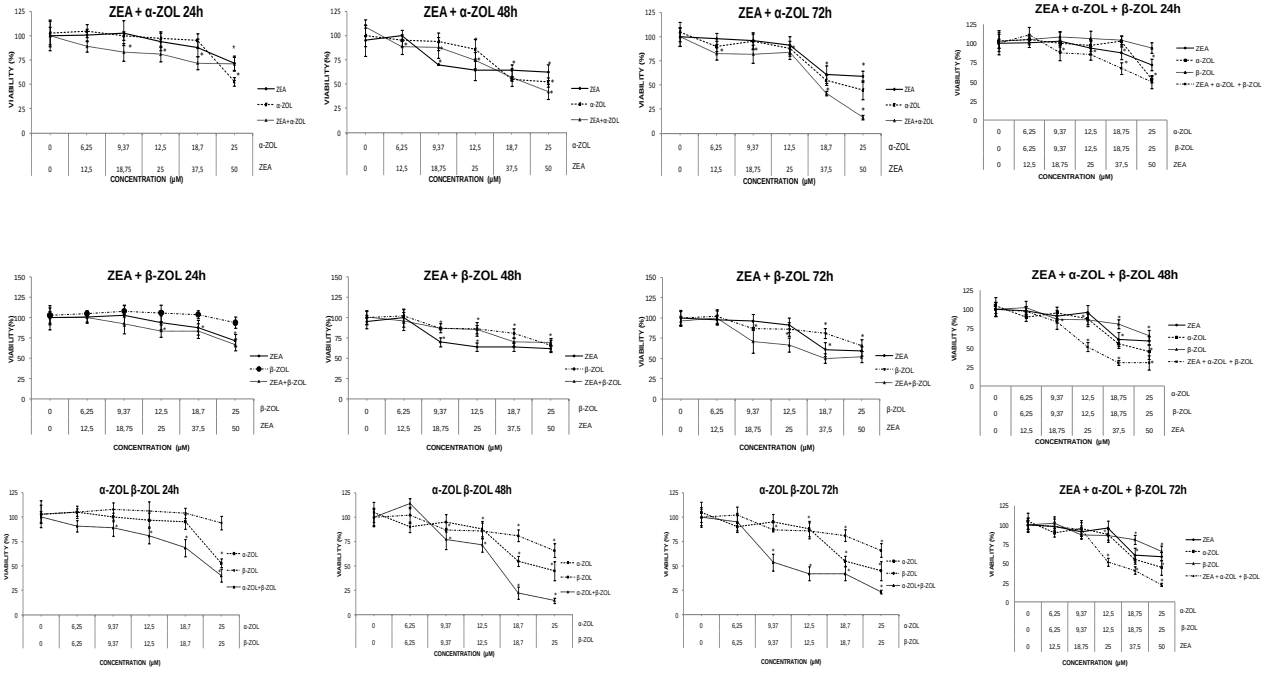
For the combination of ZEA + β-ZOL at 24 h of exposure a decreased 27% in cell viability was observed respect to β-ZOL individually tested. After 48h and 72h of exposure, this combination reduces the cell viability, approximately 30% respect to the mycotoxins tested individually (Figure 15).

The combination α-ZOL + β-ZOL (Fig. 15), at 24h of exposure showed a decrease in cell viability about 60% and 10% at the highest concentration tested compared to the viability of HepG2 cells exposed to β-ZOL and α-ZOL alone, respectively. Similarly, at 48 and 72h a decrease in cell viability at the highest concentration tested of approximately of 30% and 50% was observed compared to α-ZOL and β-ZOL tested alone, respectively.

Figure 15 shows the concentration-response curves for the tertiary combination of mycotoxins at 24, 48 and 72h of exposure on HepG2 cells. At 24h of exposure, cell viability decreased approximately 15% and 40% compared to the β-ZOL and ZEA individually tested, respectively. After 48 h of exposure, the cell viability decreased 30% compared to β-ZOL and ZEA

tested individually and approximately 15% respect to α-ZOL tested individually. At 72 h of exposure, the cell viability of tertiary combination decreased 35% compared to the ZEA and β-ZOL and 20% respect to α-ZOL tested alone.

Figure 15. Concentration-response curves for individual ZEA (—◆—), α-ZOL (---■---), β-ZOL (·–·●·–·) binary combinations (···▲···) and tertiary combinations (·–·✶·–·) of them in HepG2 cells after 24, 48 and 72 h of exposure. Concentration –response curves were fitted by non-linear regression. Data are expressed as mean value ± SD of independent experiments (n=3). $p \leq 0.05$ (*) represent significant difference from control.



The isobologram assay was used to determine the type of interaction that occurs when mycotoxins are in combination (Fig. 16 and Table 13). At 24h and 48h the ZEA + α-ZOL combination shows antagonism effect at high fractions affected and synergism at low fractions affected. Whereas, at 72h of exposure synergism effect was observed independentment of the fractional effect. The mixture of ZEA and β-ZOL showed synergistic effect at lower fractional effect and additive effect at higher fractional effect. However the effect was reverse at 72h of exposure. Synergism effects at all time and concentrations tested of exposure for α-ZOL + β-ZOL were demonstrated (Fig. 16 and Table 13). The tertiary combination (ZEA + α-ZOL + β-ZOL) showed antagonism effect at lower fractional effect which torn into additive effect at higher fractional effect at 48 and 72h of exposure.

Figure 16. Combination index (CI)/fractional effect curve as described by Chou and Talalay model for HepG2 cell exposed to ZEA, α-ZOL and β-ZOL binary and tertiary combiantions. Each point represents the CI ± SD at a fractional effect as determied in our experiments. The dotted line indicates additivity, the area under the dotted line synergy, and the area above of the dotted line antagonism. HepG2 cells were exposed during 24, 48 and 72 h with ZEA+α-ZOL and ZEA+β-ZOL at molar ratio of 2:1; α-ZOL+β-ZOL at molar ratio of 1:1 (equimolar proportion) and ZEA+α-ZOL+β-ZOL at molar ratio of 2:1:1.

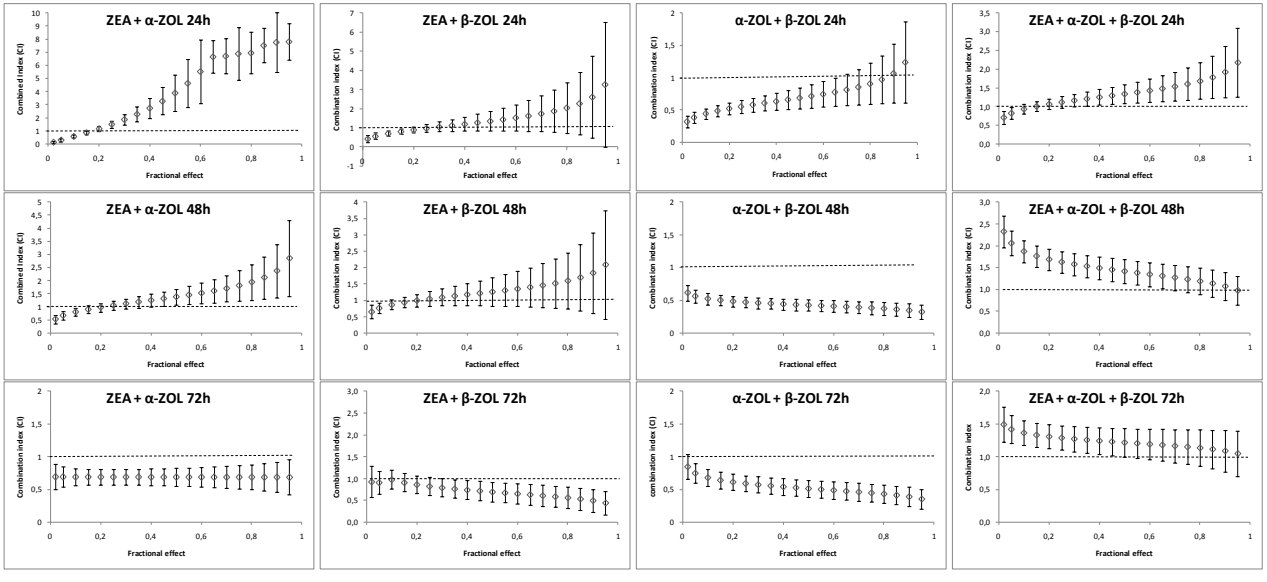


Table 13. The parameters m, Dm and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose–effect curve, the potency (IC50), and the conformity of the data to the mass-action law, respectively. Dm and m values are used for calculating the CI value (CI < 1, =1, and > 1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. IC_{25}, IC_{50}, IC_{75} and IC_{90}, are the doses required to inhibit proliferation 25, 50, 75 and 90%, respectively. CalcuSyn Software provides automatically the IC_{25}, IC_{50}, IC_{75}, IC_{90} values.

MYCOTOXIN	TIME (h)	*Dm* (μM)	*m*	*r*	CI VALUES			
					CI_{25}	CI_{50}	CI_{75}	CI_{90}
ZEA	24h	87.03020	3.27969	0.86944				
	48h	63.41079	0.77688	0.91966				
	72h	72.74993	1.91325	0.87516				
α-ZOL	24h	53.27594	3.30527	0.86834				
	48h	24.51993	2.28790	0.97890				
	72h	37.87019	1.37203	0.90647				
β-ZOL	24h	110.3766	2.78883	0.93588				
	48h	45.84811	2.11253	0.85039				
	72h	39.41671	2.18916	0.77212				
ZEA + α-ZOL	24h	186.63	0.85	0.95826	1.50 ± 0.27 Ant	3.89 ± 1.38 Ant	6.90 ± 2.00 Ant	7.77 ± 2.27 Ant
	48h	65.75	1.81	0.96024	1.05 ± 0.17 Add	1.37 ± 0.30 Add	1.80 ± 0.57 Ant	2.36 ± 1.07 Ant
	72h	32.95	3.30	0.96332	0.68 ± 0.11 Syn	0.68 ± 0.14 Syn	0.68 ± 0.17 Syn	0.68 ± 0.22 Syn
ZEA + β-ZOL	24h	84.66	1.61	0.92582	0.97 ± 0.20 Add	1.35 ± 0.51 Add	1.88 ± 1.10 Add	2.61 ± 2.15 Add
	48h	78.74	2.04	0.93590	1.05 ± 0.17 Add	1.26 ± 0.42 Add	1.52 ± 0.75 Add	1.83 ± 1.22 Add
	72h	43.37	6.05	0.82670	0.82 ± 0.20 Add	0.69 ± 0.22 Syn	0.58 ± 0.24 Syn	0.49 ± 0.25 Add
α-ZOL + β-ZOL	24h	24.57	1.91	0.95791	0.54 ± 0.09 Syn	0.68 ± 0.27 Syn	1.06 ± 0.45 Add	1.06 ± 0.45 Add
	48h	15.20	4.40	0.96730	0.46 ± 0.08 Syn	0.38 ± 0.09 Syn	0.34 ± 0.09 Syn	0.34 ± 0.10 Syn
	72h	18.43	5.15	0.90667	0.59 ± 0.12 Syn	0.44 ± 0.13 Syn	0.44 ± 0.13 Syn	0.38 ± 0.14 Syn
ZEA + α-ZOL + β-ZOL	24h	52.68	2.08	0.97732	1.11 ± 1.15 Add	1.33 ± 0.25 Add	1.60 ± 0.42 Ant	1.92 ± 0.68 Ant
	48h	55.56	5.34	0.96128	1.62 ± 0.24 Ant	1.41 ± 0.26 Ant	1.22 ± 0.29 Add	1.07 ± 0.29 Add
	72h	47.83	3.81	0.96892	1.29 ± 0.18 Ant	1.21 ± 0.22 Add	1.14 ± 0.26 Add	1.08 ± 0.31 Add

3.3 Degradation of ZEA and metabolites generated

The separation of ZEA and its metabolites was achieved by LC/MS/LIT. The linearity in LC–MS/MS was obtained in triplicate by spiking 6 concentrations (1, 10, 50, 100, 250 and 500 µM). The linear regression coefficient of calibration curves showed a correlation coefficient (r2) higher than 0.992. Recovery experiments were conducted at two different levels, one between 5 and 50 µM (LQs) and the other between 50 and 500 µM (10 times LQs). In both cases, mycotoxins added before the corresponding extraction procedure evidenced a recovery ranged from 75 to 99%. The values of intra-day precision (n = 6) and inter-day precision (n = 6) were inferior to 10% and 14%, respectively. Sensitivity was evaluated by LD and LQ values. The LD was estimated from blank extract, spiked with decreasing concentrations of the analytes. The response of the mycotoxin peak was equal to three times the response of the blank sample (n=20). Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LD and LQ evidenced for the mycotoxins studied ranged from 1 to 5 µM.

To determine the metabolic capacity of HepG2 cells on ZEA and its metabolites, the HepG2 cells were exposed to 50 µM of each mycotoxins during 24 and 48h. To detect the metabolic change of ZEA, α-ZOL and β-ZOL, the growth medium of the cells exposed to these mycotoxins was collected and injected in the LC-MS-LIT. The growth medium exposed to these mycotoxins without cells was considered as control. The concentrations of ZEA and its metabolites decreased in the grow medium from 77.6±1.0 to 70.1±0.8 for ZEA; from 66.2±1.1 to 64.7±1.6 for α-ZOL and from 46.8±1.2 to 37.0±0.9 for β-ZOL at 24 and 48h of exposure. The mycotoxins were not detected inside the cells.

The Table 14 shows the degradation products obtained at 24 and 48 h for ZEA and its metabolites in the growth medium. Worth highlighting the formation of deshydroxylation products in both ZEA and its metabolites in growth medium. The Table 4 shows the degradation products of ZEA and its metabolites in cells.

Table 14. Degradation products of ZEA, α-ZOL and β-ZOL at 24 and 48h in the growth medium when HepG2 cells were exposed to 50μM of these mycotoxins.

PRODUCT	24h			48h		
	ZEA (μM)	α-ZOL (μM)	β-ZOL (μM)	ZEA (μM)	α-ZOL (μM)	β-ZOL (μM)
demethylation	0.00	0.67	0.78	1.53	0.69	1.06
dehydroxylation (-1OH)1	**45.30**	**8.67**	**8.51**	**52.27**	**6.78**	**10.63**
dehydroyilation (-1OH) 2	0.00	0.50	0.64	0.00	0.58	0.43
dehydroxylation (-2OH) 1	9.76	0.58	0.25	13.94	0.54	0.18
dehydroxylation (-2OH) 2	0.00	0.00	0.51	0.00	0.00	0.40
hydroxylation (+1OH)	0.00	7.88	0.62	0.00	12.61	0.11
glucosidation (+1G)	0.00	3.23	1.70	0.00	2.36	1.98
glucosidation (+2G)	0.00	0.67	0.18	0.45	0.00	0.11
glucuronation	0.56	0.00	0.00	2.89	0.00	0.00
16-glucose	3.41	0.00	0.00	3.48	0.00	0.00
	59.03	22.21	13.18	74.57	23.55	14.91

4 Discussion

In order to evaluate the combined effects between the ZEA and its metabolites (α-ZOL, β-ZOL), it is necessary to determinate the cytotoxic effect which occurs when these mycotoxins are tested alone. For that, HepG2 cells were exposed to different concentrations of these mycotoxins. The results shown that cells viability decreases as the concentration of mycotoxins increased in the following order α-ZOL > β-ZOL > ZEA. The results obtained in this study are similar to those obtained by Marin [7] that demonstrated that in PMNs cells ZEA was less cytotoxic than its

metabolites. On the other hand, Ayed [4] demonstrated that the ZEA is more cytotoxic than its metabolites, when they exposed HeLa cells to these mycotoxins for 24h. Bibliografic data obtained from different authors demonstrated that the difference in the type of mycotoxins mixtures, the method used for analyzing the concentration effect relationship, period of exposure, concentration range tested and the type of cells produce different results.

Respect to cytotoxicity of mycotoxins mixtures, binary combinations of different mycotoxins are widely studied. However, only a few studies determine the cytotoxicity produced by tertiary combination of mycotoxins. Kouadio [13] studied the cytotoxicity that occurred when Caco-2 cells were exposed to a mixture of FB1 + DON + ZEA. In this study, the RN method was used to determine both, the cytotoxicity caused by mycotoxins individually and the cytotoxicity produced by binary and tertiary combinations. These authors obtained that the tertiary combination shows a synergism effect. Ruiz [24,25] used the isobologram method to determine whether the cytotoxicity produced by the mixture of BEA + ZEA + PAT caused additive synergism or antagonist effect when compared to the cells exposed to mycotoxins individually. These authors observed a synergism effect on CHO-K1 cells and antagonism effect on Vero cells. Klaric [26] evidenced an increase in LDH activity when compared the activity of LDH that produced the FB1, BEA and OTA alone and their mixtures which showed antagonis effect. Tatay [17] used the mixture of ZEA + α-ZOL + β-ZOL on CHO-K1 cells. Their results shown antagonism effects at high fractions affected and synergism effect at low fractions affected.

With the isobologram method is difficult to explain this different behavior of mycotoxins in combination, since this method only allows quantitative determinations of synergism, additive or antagonism effect but not the

mechanism whereby these interactions occurs. However, the additive effect showed by ZEA and its metabolites can be explained by the similar structure of these mycotoxins may compete with the same receptor site.

Related to biotransformation pathways of ZEA in HepG2 cells, it is possible to hypothesized that biotransformation of ZEA occurs in two major pathways: 1-Hydroxyation resulting in the two major phase I metabolites (α-ZOL and β-ZOL). 2- The conjugation of ZEA and its reduced metabolites with glucuronic acid (phase II) [27, 28, 29].

Results obtained in this study shown that HepG2 cells cannot metabolize ZEA into α-ZOL and β-ZOL but other degradation products are formed. Some of these products were found previously [17, 28, 29, 30, 31]. [28] demostrated the formation of several glucuronides and sulfates when Caco-2 cells were incubated with ZEA. [28] determinated the in vivo metabolism of ZEA through the analysis of urine samples obtained from voluntaries demonstrating the urinary excretion of ZEA-14-glucuronide. Similarly, our results shown some glucuronide products when HepG2 cells were trated with α-ZOL and β-ZOL. [30] Studied the metabolism and the transfer of ZEA in Caco-2 cells as a model of intestinal epithelium. The cells were exposed to ZEA (10-100 μM) evidencing an efficacious metabolism of the ZEA into α-ZOL and β-ZOL. [27] treated Caco-2 cells with ZEA and after an incubation of 24h analyzed the samples demonstrating that neither ZEA or its metabolites were detected in the samples. In our studied ZEA and its metabolites are detected in the medium but not inside the cells. [17] demostrated the formation of deshydroxylation products when CHO-K1 cells were treated with α-ZOL. This study shown the formation of dehydroxylation products when HepG2 cells are exposed to ZEA and its metabolites.

In conclusion this extraction procedure was adequate to know the concentration of these type of mycotoxins in mammalian cells. The confirmation technique (LC-MS-LIT) could help to know ZEA α-ZOL and β-ZOL and their degradation products which can contribute to cytotoxicity in HepG2 cells. And the MTT assay is a sensitive method suitable to determinate the cytotoxicity when these mycotoxins are individually and in combination. So these results obtained for ZEA α-ZOL and β-ZOL by *in vitro* toxicity can help to better understaning the behavior of mycotoxins in *in vivo* study.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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3.3 Cytotoxic effects of zearalenone and its metabolites and antioxidant cell defense in CHO-K1 cells

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Cytotoxic effects of zearalenone and its metabolites and antioxidant cell defense in CHO-K1 cells

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Abstract

Zearalenone (ZEA) and its metabolites (α-zearalenol; α-ZOL, β-zearalenol; β-ZOL) are secondary metabolites of *Fusarium* fungi that produce cell injury. The present study explores mycotoxin-induced cell damage and cellular protection mechanisms in CHO-K1 cells. Cytotoxicity has been determined by reactive oxygen species (ROS) production and DNA damage. ROS production was determined using the fluorescein assay and DNA strand breakage by comet assay. Intracellular protection systems were glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD). The results demonstrated that all mycotoxins increased the ROS levels up to 5.3-fold the control levels in CHO-K1 cells. Zearalenone metabolites, but not ZEA, increased DNA damage 43% (α-ZOL) and 28% (β-ZOL) compared to control cells. The GSH levels decreased from 18% to 36%. The GPx and SOD activities respectively increased from 26% to 62% and from 23% to 69% in CHO-K1 cells, whereas CAT activity decreased from 14% to 52%. In addition, intracellular ROS production was induced by ZEA and its metabolites. The endogenous

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antioxidant system components GSH, GPx and SOD were activated against ZEA and its metabolites. These antioxidant system components thus could contribute to decrease cell injury by ZEA and its metabolites.
Keywords: Zearalenone; ZEA metabolites; ROS production; DNA damage; Antioxidant defense system

1. Introduction

Mycotoxins represent one of the most important categories of natural toxins. Exposure occurs through the ingestion of food contaminated by mycotoxins, causing diseases in human and animals. These diseases can be acute or chronic depending on the dose, time of exposure and potential toxicity of the mycotoxins. The Food and Agriculture Organization of the United Nations (FAO) estimates that 25% of the cereals produced in the world are contaminated by mycotoxins. Furthermore, mycotoxins in food have become a serious human concern and may cause significant economic losses (FAO, 2013).

Zearalenone (ZEA) and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) are secondary metabolites of *Fusarium* fungi, which can produce serious health problems. They elicit an estrogenic response by mimicking sex steroid hormones, because they share similar molecular mechanisms and activity with natural estrogens. These metabolites of *Fusarium* fungi interact with the estrogen receptors (ERα and ERβ), causing functional and morphological changes in the reproductive system of animals and humans (Pazaiti et al., 2011). Zearalenone can produce reactive oxygen species (ROS) in mammalian cells (Ferrer et al., 2009). Cells have cellular protection mechanisms against biological reactive intermediates, xenobiotics (including mycotoxins) and metabolic products. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase

(GPx) are important defense enzymes for cell survival in the face of oxidative stress, and glutathione (GSH) affords antioxidant protection against oxidative events (Deponte, 2013). When there is an imbalance between the production of oxidizing molecular species and the presence of cellular antioxidant agents in favor of the pro-oxidants, changes in cellular signals and certain types of damage may result. The targets for oxidative species are mainly DNA, proteins and lipids (Hassen et al., 2007). Zearalenone impairs cell proliferation through apoptotic cell death; inhibition of DNA and protein synthesis; DNA fragmentation; the production of micronuclei, sister chromatid exchanges (SCEs) and chromosomal aberrations (CAs); the production of DNA adducts; and changes in the mitotic index (MI), etc. (Kouadio et al., 2005; Zinedine et al., 2007; Abid-Essefi et al., 2009; Marin et al., 2010; Lu et al., 2013 ; Venkataramana et al., 2014). The level of strand breaks in DNA is an indicator of genotoxicity, because these are common lesions produced by a wide range of agents and involving diverse mechanisms. Comet assaying is the most popular method for measuring several types of DNA damage, including oxidative damage produced by ROS (Collins, 2014). The Comet assay is widely used, since it requires a small number of cells and a short time to complete the study. Besides, compared with other assays, this method has several advantages, since it is sensitive in detecting low levels of DNA damage, easy to apply, flexible, inexpensive, and requires little time to complete the experiment (Kang et al., 2013).

Many studies have been published on the cytotoxic effects and cellular defense systems related to ZEA exposure in mammalian cell cultures. However, there is little information on its metabolites, and it is important to have a global vision of both the damage and antioxidant processes in order to determine whether the adaptive response of the cell is sufficient, as well

as to know whether the metabolites are more or less toxic than their precursor. The aims of this study were to determine ROS generation and DNA damage produced by ZEA and its metabolites, and to analyze the antioxidant defense mechanisms (GSH, GPx, CAT and SOD) against the cytotoxic effects of these mycotoxins in CHO-K1 cells.

2. Material and methods

2.1. Reagents

The reagent grade chemicals and cell culture components used, penicillin, streptomycin, HEPES, phosphate buffer saline (PBS), glucose, dimethyl sulfoxide (DMSO), triton X-100, 2′,7′-dichlorodihidrofluorescein diacetate (H_2-DCFDA), *o*-phthaldialdehyde (OPT), propidium iodide (PI), Na-EDTA, agarose, low melting point agarose (LMA) and analytical standard of mycotoxins were from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Cambrex Co. (Belgium). Deionized water (resistivity < 18 MΩ cm) was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). Mycotoxins selected, ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol), were from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of mycotoxins were prepared in methanol and maintained at −20 °C in the dark. The final concentrations tested were achieved by adding the culture medium with mycotoxins, and the final methanol concentration in medium was ≤1% (v/v). These concentrations have been proven to be non-toxic.

2.2. Cell culture and exposure to ZEA and its metabolites

Cells were grown at 37 °C in 9 cm^2 polystyrene tissue culture dishes. CHO-K1 cells were grown using Ham's-F12 medium. Media were

supplemented with 25 mM HEPES buffer (pH 7.4), 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The incubation conditions were 5% CO_2 at 37 °C and 95% air atmosphere at constant humidity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

For all assays, three different concentrations, 6.25, 12.5 and 25 μM, of ZEA and its metabolites were selected according to previous outcomes (these concentrations being lower than the IC_{50} values obtained in a previous study) (Tatay et al., 2014a). Tatay et al. (2014a) demonstrated that after 24 h of exposure, the IC_{50} values were >100, 33.00 ± 4.50 and >75 μM for ZEA, α-ZOL and β-ZOL, respectively. After 48 h of exposure, the IC_{50} values were 60.30 ± 5.99, 32.00 ± 5.15 and 62.50 ± 4.56 μM for ZEA, α-ZOL and β-ZOL, respectively. In turn, after 72 h of exposure, the IC_{50} values were 68.00 ± 2.55, 30.00 ± 5.50 and 55.00 ± 3.80 μM for ZEA, α-ZOL and β-ZOL, respectively.

2.3. ROS production

Intracellular ROS production was monitored in CHO-K1 cells by adding dichlorofluorescein diacetate (DCF-DA). This method is exceptionally sensitive and provides a direct measure of overall oxidative stress, with the detection of intracellular oxidants. DCF-DA is taken up by the cells and then deacetylated by intracellular esterases - the resulting H_2-DCFDA becoming trapped inside the cell. Oxidation by ROS of the non-fluorescent moiety H_2-DCFDA to highly fluorescent dichlorofluorescein (DCF) can occur. The method was carried out according to Ruiz-Leal and George (2004). Briefly, 2×10^4 cells/well were seeded in a 96-well black culture microplate in Ham's-F12 medium. Once the cells reached 65% confluence, the culture medium was replaced and the cells were loaded with 20 μM DCFH-DA for

20 min. The medium with DCFH-DA was then removed and washing with PBS was performed twice before the addition of medium with 1% MeOH (control), medium with ZEA or medium with its metabolites. Increases in fluorescence were measured at intervals up to 2 h at excitation and emission wavelengths of 485 and 535 nm, respectively. Twenty-four replicates were developed. Results are expressed as the increase in fluorescence with respect to the control. Determinations were performed in three independent experiments.

2.4. Alkaline comet assay

CHO-K1 cells were plated in 6-well tissue culture plates at a density of 3.4×10^5 cells per well and grown to confluence overnight. The growth medium was removed and ZEA and its metabolites (α-ZOL and β-ZOL), at the concentrations of 6.25, 12.5 and 25 μM, were added to the medium, allowing uptake to proceed for 24 h. The alkaline comet assay was performed to determine DNA strand breaks according to the method described by Mallebrera et al. (2016). Results are expressed as the percentage of DNA in tail (%), calculated according to the equation: % DNA in tail = (total intensity of tail/total intensity of comet) x 100. Total intensity of comet = head length + tail length. Determinations were performed in three independent experiments.

2.5. GSH determination

For determining the GSH levels and GPx, SOD and CAT activities in CHO-K1 cells exposed the ZEA and its metabolites, 3×10^5 cells/well were seeded in 6-well culture plates. After the cells reached confluence, the culture medium was removed and the cells were treated with ZEA and its metabolites (6.25, 12.5 and 25 μM) for 24 h. Then, the medium was

removed and the cells were washed twice with PBS and homogenized in 0.5 ml of 20 mM Tris and 0.1% Triton.

Determination of reduced GSH was assayed by adapting the method of Maran et al. (2009). Briefly, 10 µl of each homogenized cell sample was placed in a 96-well black tissue culture plate with 200 µl GSH buffer (0.1 M phosphate 5 mM EDTA pH 8.0) and 10 µl of OPT solution (1 mg/ml absolute methanol), mixed and incubated in darkness at room temperature for 15 min. The concentration of GSH was determined using a microplate reader (Wallace Victor2, 1420 Multilabel Counter, Perkin Elmer, Turku, Finland) with excitation and emission wavelengths of 345 and 425 nm, respectively. GSH levels were expressed as µg GSH/mg protein. Determinations were performed in triplicate.

2.6. Determination of enzyme activities

GPx activity was assayed spectrophotometrically using H_2O_2 as substrate for the Se-dependent peroxidase activity of GPx by following oxidation of NADPH at 340 nm during the first 2 min in a coupled enzymatic reaction with GR, as described by Maran et al. (2009). In 1 ml final volume, the reaction mixture contained 500 µl of 0.1 M phosphate buffer (pH 7.5, 1 mM EDTA and 2 mM NaN_3, 0.1% Triton X-100), 250 µl of ultrapure water, 100 µl of 20 mM GSH, 20 µl of 0.2 mM NADPH, 2.5 U of freshly prepared GR, and 50 µl of 5 mM H_2O_2. Fifty µl of homogenized cell sample was added to the reaction mixture. One unit of GPx will reduce 1 µmol of GSSG per min at pH 7.5. Assays were conducted at 25 °C during 2 min in a thermocirculation Perkin Elmer UV–VIS spectrometer Lambda 2 version 5.1. GPx enzymatic activity was calculated by using the molar absorptivity of NADPH (6.22 mM^{-1} cm^{-1}), and expressed as nmol of NADPH oxidized/min/mg of protein. Determinations were performed in triplicate.

SOD activity was determined with the Ransod kit (Randox Laboratories, United Kingdom) adapted for 1.5 ml cuvettes. SOD activity was monitored at 505 nm during 3 min at 37 °C in a thermocirculation Perkin Elmer UV–VIS spectrometer Lambda 2 version 5.1. SOD enzymatic activity was expressed as units of SOD per mg protein. Determinations were performed in triplicate.

CAT activity was measured according to Espín et al. (2014), with slight modifications. Briefly, 50 µl of the homogenized cell suspension was mixed with 950 µl of 0.05 M NaH_2PO_4 and 500 µl of 0.03 M of H_2O_2. The kinetics of the enzymatic decomposition of H_2O_2 were determined as absorbance decrements at 240 nm for 2 min with a spectrophotometer (Super Aquarius CECIL CE 9500). CAT enzymatic activity was calculated by using the molar absorptivity of H_2O_2 (43.6 mM^{-1} cm^{-1}), and expressed as µmol of H_2O_2/min/mg of protein. Determinations were performed in triplicate.

2.7. Determination of total protein content

Cellular protein content was assayed using Bio-Rad DC Protein Assay; catalog number 500-0116 (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf). Protein concentration was measured at 690 nm.

2.8. Statistical analyses

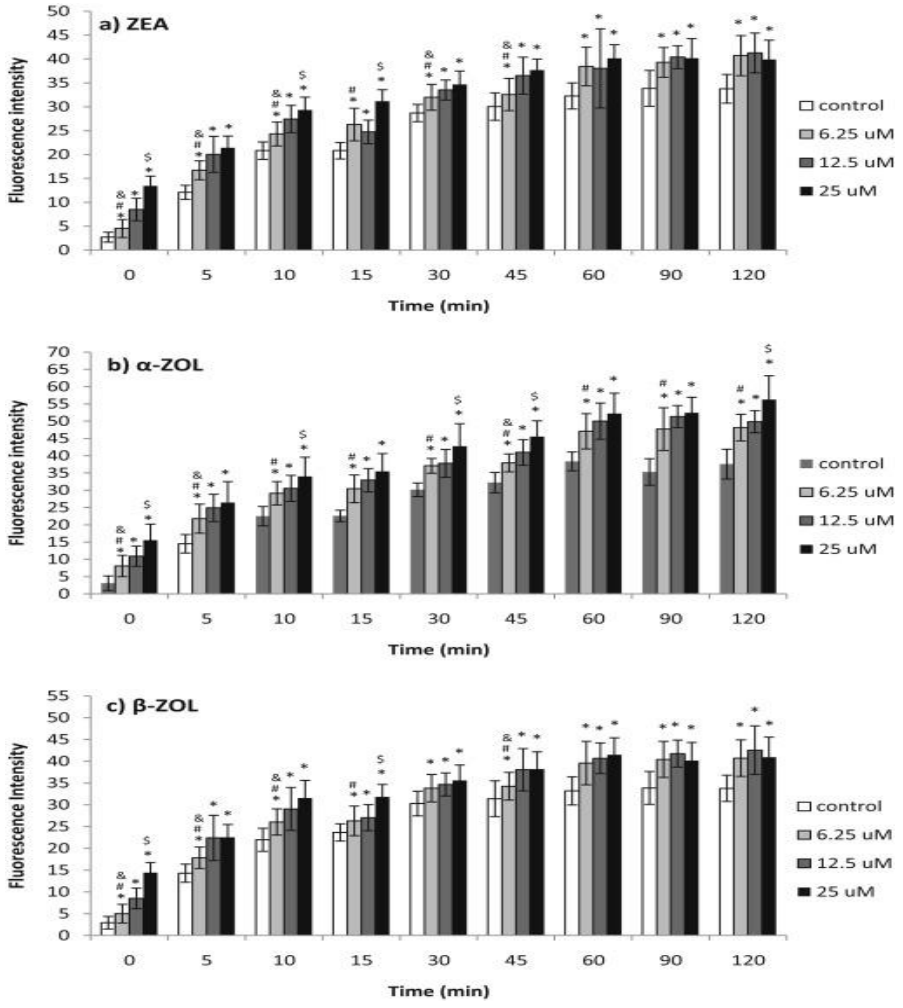
Statistical analysis of the data was carried out using the SPSS version 19.0 statistical package (SPSS, Chicago, IL, USA). Data were expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for *post hoc* comparisons. Statistical significance was considered for $p \leq 0.05$.

3. Results

3.1. ROS production

To determine changes in oxidative damage, CHO-K1 cells were exposed to different concentrations of ZEA and its metabolites from 0 to 120 min. The results obtained by the fluorescein assay (Fig. 17) demonstrated an increase in the concentration of oxidizing species in CHO-K1 cells exposed to ZEA and its metabolites compared to the basal rate. Reactive oxygen species production was time- and concentration-dependent for α-ZOL at all concentrations tested (Fig. 17b), while for ZEA and β-ZOL, time and concentration dependence were observed from 0 min to 45 min of exposure (Fig. 17a and c). The highest intensity of fluorescence in CHO-K1 cells was observed at the shorter exposure times compared to controls. In the presence of ZEA, α-ZOL and β-ZOL, the ROS levels increased 4.3-, 5.3- and 4.6-fold the control level, respectively, at 0 min. However, at the longest time tested, ROS production did not exceed 1.7-fold the control level in the presence of ZEA and its metabolites (Fig. 17).

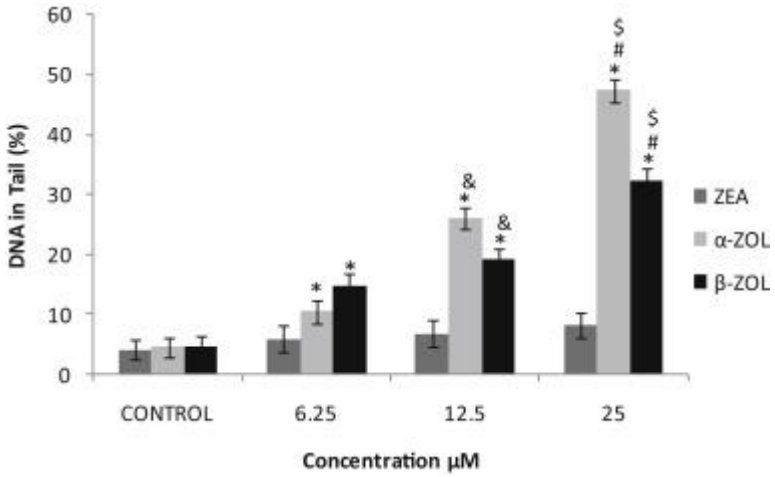
Fig. 17. Time dependence of ROS-induced fluorescence in CHO-K1 cells exposed to ZEA, α-ZOL and β-ZOL. CHO-K1 cells were exposed to H_2-DCFDA for 20 min before ZEA, α-ZOL and β-ZOL. Results are expressed as mean ± SEM (n = 3). (*) $p \leq 0.05$ indicates significantly different from control. (&) $p \leq 0.05$ indicates significant differences between 6.25 and 12.5 μM concentrations. (#) $p \leq 0.05$ indicates significant differences between 6.25 and 25 μM concentrations. ($) $p \leq 0.05$ indicates significant differences between 12.5 and 25 μM concentrations.



3.2. Comet assay

The comet assay was used to determine direct DNA damage after cell exposure to ZEA, α-ZOL and β-ZOL (Fig. 18). DNA damage was observed after ZEA metabolites incubation in CHO-K1 cells. Zearalenone did not produce DNA damage. An increase was observed in the proportion of DNA in the tail as the α-ZOL and β-ZOL concentrations increased. α-ZOL produced greater damage to cellular DNA (43%) than β-ZOL (28%) (Fig. 18). Significant differences were observed for α-ZOL at all concentrations tested (Fig.18). Significant differences between 6.25 *vs.* 12.5 μM (approximately 149-fold), between 6.25 *vs.* 25 μM (approximately 354-fold), and between 12.5 *vs.* 25 μM (approximately 82-fold) were observed when CHO-K1 cells were exposed to α-ZOL (Fig. 18). No differences between 6.25 and 12.5 μM β-ZOL were observed related to DNA damage in CHO-K1 cells (Fig. 18). However, significant differences in DNA damage were observed for β-ZOL between 6.25 *vs.* 25 μM (approximately 118-fold) and between 12.5 *vs.* 25 μM (approximately 68-fold) (Fig. 18).

Fig. 18. Percentage (%) of DNA in tail in CHO-K1 cells after exposure to 6.25, 12.5 and 25 μM of ZEA, α-ZOL and β-ZOL during 24 h. Data are expressed as mean value ± SEM (n = 3). (*) $p \leq 0.05$ indicates significant differences from control. (&) $p \leq 0.05$ indicates significant differences between 6.25 and 12.5 μM concentrations. (#) $p \leq 0.05$ indicates significant differences between 6.25 and 25 μM concentrations. ($) $p \leq 0.05$ indicates significant differences between 12.5 and 25 μM concentrations.

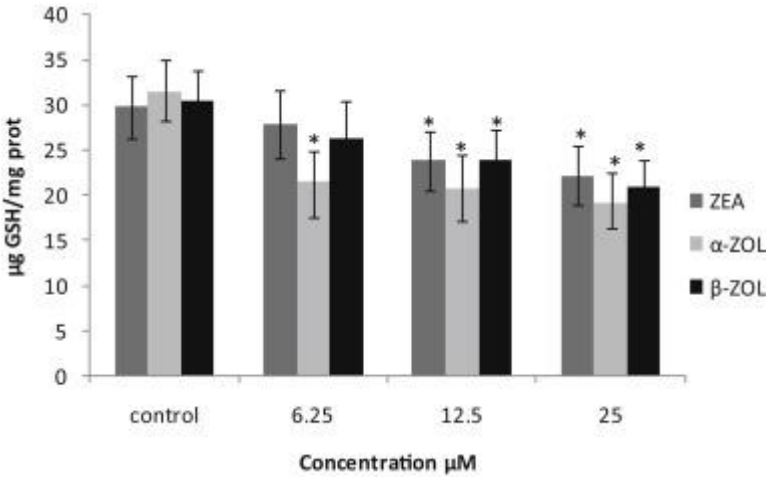


3.3. Determination of GSH levels and enzyme activities

Measurements were made of the GSH levels when CHO-K1 cells were exposed to 6.25, 12.5 and 25 μM of ZEA and its metabolites. The GSH levels were not affected at 6.25 μM of ZEA and β-ZOL. However, following exposure to 12.5 and 25 μM of ZEA and its metabolites, the GSH levels decreased significantly by 20% and 26% (ZEA), 25% and 36% (α-ZOL) and 28% and 18% (β-ZOL), *versus* their own controls in CHO-K1 cells, respectively (Fig. 19). A greater decrease in GSH levels was observed after α-ZOL exposure in CHO-K1 cells *versus* the control. No differences related

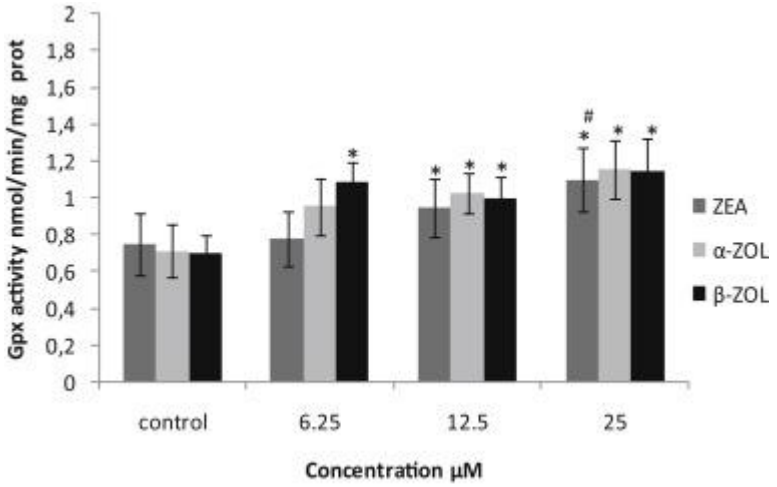
to GSH levels between all mycotoxins at 12.5 or 25 μM were observed (Fig. 19).

Fig. 19. GSH levels in CHO-K1 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) during 24 h of incubation. Data are expressed as mean value ± SEM (n = 3). (*) $p \leq 0.05$ indicates significant differences from control.



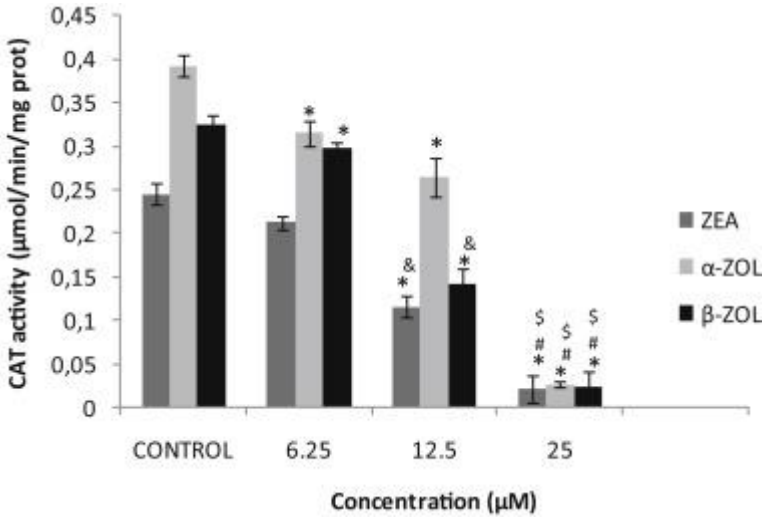
GPx activity increased significantly upon exposure to 12.5 and 25 μM of ZEA and its metabolites in CHO-K1 cells (Fig. 20). After CHO-K1 cells were exposed to 12.5 and 25 μM (ZEA, α-ZOL and β-ZOL), GPx activity increased from 26% to 49%, from 44% to 62%, and from 43% to 61%, respectively, *versus* the controls. No changes in GPx activity were observed at 6.25 μM of ZEA or α-ZOL. On the other hand, we observed a significant difference (approximately 41%) between 6.25 and 25 μM of ZEA (Fig. 20).

Fig. 20. GPx activity in CHO-K1 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) after 24 h of incubation. Data are expressed as mean value ± SEM (n = 3). (*) $p \leq 0.05$ indicates significant differences from control. (#) $p \leq 0.05$ indicates significant differences between 6.25 and 25 μM concentrations.



CAT activity was evaluated by the depletion in H_2O_2 levels after CHO-K1 cells were treated with 6.25, 12.5 and 25 μM of ZEA and its metabolites (Fig. 21). CAT activity decreased from 21% to 34% with ZEA, from 26% to 52% with α-ZOL, and from 14% to 42% with β-ZOL in CHO-K1 cells. α-ZOL showed greater effectiveness in decreasing CAT activity in CHO-K1 cells (Fig. 21). CAT activity decreased significantly when comparing 6.25 *vs.* 25 μM (91%), and 12.5 *vs.* 25 μM (88%) for α-ZOL (Fig.21). No difference between 6.25 and 12.5 μM of α-ZOL was observed (Fig. 21). Both ZEA and β-ZOL significantly reduced CAT activity between 43% and 53% (6.25 *vs.* 12.5 μM), 83% and 86% (12.5 *vs.* 25 μM), and 90% and 93% (6.25 *vs.* 25 μM) (Fig. 21).

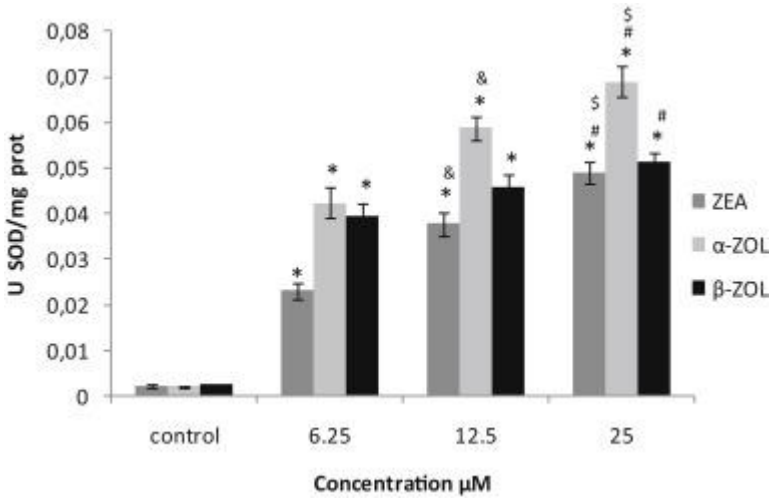
Fig. 21. CAT activity in CHO-K1 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) during 24 h of incubation. Data are expressed as mean value ± SEM (n = 3). (*) $p \leq 0.05$ indicates significant differences from control. (&) $p \leq 0.05$ indicates significant differences between 6.25 and 12.5 μM concentrations. (#) $p \leq 0.05$ indicates significant differences between 6.25 and 25 μM concentrations. ($) $p \leq 0.05$ indicates significant differences between 12.5 and 25 μM concentrations.



SOD levels were measured after CHO-K1 cells were treated with different concentrations of ZEA and its metabolites during 24 h (Fig. 22). SOD activity increased from 23% to 48% with ZEA, from 42% to 69% with α-ZOL, and from 39% to 51% with β-ZOL in CHO-K1 cells. Higher SOD activity was observed after α-ZOL exposure in CHO-K1 cells (Fig. 22). SOD activity increased significantly on comparing 6.25 *vs.* 12.5 μM (65%), 6.25 *vs.* 25 μM (113%) and 12.5 *vs.* 25 μM (28%) for ZEA (Fig. 22). SOD activity increased significantly on comparing 6.25 *vs.* 12.5 μM (40%), 6.25 *vs.* 25 μM (64%) and 12.5 *vs.* 25 μM (16%) for α-ZOL (Fig. 22). β-ZOL

significantly increased SOD activity by approximately 28% (6.25 *vs.* 25 μM). No difference in SOD activity was observed between 6.25 and 12.5 μM or between 12.5 and 25 μM of β-ZOL (Fig. 22).

Fig. 22. SOD activity in CHO-K1 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) during 24 h of incubation. Data are expressed as mean value ± SEM (n = 3). (*) $p \leq 0.05$ indicates significant difference from control. (&) $p \leq 0.05$ indicates significant differences between 6.25 and 12.5 μM concentrations. (#) $p \leq 0.05$ indicates significant differences between 6.25 and 25 μM concentrations. ($) $p \leq 0.05$ indicates significant differences between 12.5 and 25 μM concentrations.



4. Discussion

In previous studies conducted in our laboratory, ZEA and its metabolites were seen to cause cytotoxic effects and oxidative stress in CHO-K1 and HepG2 cells (Ferrer et al., 2009; Tatay et al., 2014a ; Tatay et al., 2014b). Taking into account that ROS generation can be a cause or consequence

of mitochondrial alterations, these mycotoxins can damage cell function and/or structure. Accordingly, the present study aimed to determine the mechanisms whereby ZEA and its metabolites induce cytotoxic effects in cells. These mycotoxins increase ROS generation in a time- (0–120 min) and concentration-dependent (6.25–25 μM) manner. Similarly, Ferrer et al. (2009) suggested that ZEA (1–50 μM) affects the redox status, increasing ROS production in CHO-K1 cells at an early stage of ZEA exposure. Gazzah et al. (2010) and El Golli Bennour et al. (2009) in turn demonstrated increased ROS production in a time-dependent (24, 40 and 60 h) manner in HepG2 cells. Moreover, they observed indirect oxidative status modifications after ZEA exposure. A time-dependent increase in ROS production was also reported by Venkataramana et al. (2014) in human neuroblastoma cells (SH-SY5Y) exposed to ZEA (25–200 μM), over a period of 24 h. Likewise, these authors demonstrated the protective effect of *N*-acetylcysteine (NAC) against ZEA through the neutralization of excessive ROS production. Similarly, increased ROS triggered by ZEA (5–40 μM) has been observed in mouse Leydig tumor cells (MLTC-1) and bronchial epithelial cells (BEAS-2B) (Li et al., 2014 ; So et al., 2014). Gao et al. (2013) exposed human embryonic kidney cells (HEK293) to ZEA (10–20 μM) and recorded an increase in ROS production after 2 h of treatment with respect to the control. They also showed ZEA to produce lysosomal alterations resulting in mitochondrial damage. Similar cellular dysfunctions have been reported in response to ZEA-induced (40–120 μM) oxidative damage in Vero cells (Abid-Essefi et al., 2012). Bouaziz et al. (2008) reported that 120 μM of ZEA produced an important increase in ROS generation strongly related to $O_2^{\cdot-}$ generation in HepG2 cells. Banjerdpongchai et al. (2010) in turn evidenced the ability of ZEA to generate ROS in HL-60 cells. These authors detected peroxide radicals

and other active oxygen radicals, and demonstrated that ZEA causes apoptotic cell injury via oxidative stress. All these findings, which are consistent with our own data, corroborate that ROS could be implicated in oxidative stress and consequently in cell death. In contrast, Lei et al. (2013) found that 10 μM of ZEA did not increase ROS production in PK-15 cells - though it ameliorated ROS production induced by low concentration aflatoxin B1 (1 μM). Similar results were obtained by Sun et al. (2015) after ZEA (10–40 μM) treatment in a rat liver cell line (BRL 3A) during 24 h.

Few studies have been published on ZEA metabolites and the mechanisms involved in the intracellular generation of ROS. Lu et al. (2013) observed an increase in DCF fluorescence about 1.5- and 2-fold greater than that seen in untreated cells after macrophages were treated with 50 μM of α-ZOL and β-ZOL, respectively. They suggested implication of the Haber-Weis reaction (production of hydroxyl radicals ($OH^{\bullet}$ from H_2O_2 and $O_2^{\bullet -}$) as well as the Fenton reaction (production of $OH^{\bullet}$ from H_2O_2 and iron) in the production of ROS by ZEA metabolites. Differences between ZEA metabolite mechanisms can be due to the different capacity of these metabolites to hydrolyze $H_2O_2$and scavenge hydroxyl radicals. Othmen et al. (2008) determined the effect of α-ZOL and β-ZOL upon oxidative stress by measuring malondialdehyde (MDA) in Vero cells. In concordance with Ferrer et al. (2009) following ZEA exposure, they found α-ZOL (21.25, 42.5 and 85 μM) and β-ZOL (13.75, 27.5 and 55 μM) to induce LPO in a concentration-dependent manner in Vero cells.

In order to determine whether the ROS produced by ZEA and its metabolites are implicated in DNA damage, the genotoxic effects of these mycotoxins were examined in the present study by measuring breaks in DNA strands. The results obtained evidenced an increase in DNA damage

in CHO-K1 cells exposed to α-ZOL and β-ZOL, but not after incubation with ZEA. Moreover, β-ZOL was found to be less genotoxic than α-ZOL. Other authors have revealed that ZEA can cause DNA damage in mammalian cells depending on the concentration of the mycotoxin. Kang et al. (2013) detected DNA damage in individual Chang liver cells (CCL 13) caused by ZEA (50–200 μM), with a marked increase (from 7.43 ± 0.35 to 19.01 ± 4.21) *versus* the control (0.32 ± 0.14). Hassen et al. (2007) in turn found ZEA (5–100 μM) to induce oxidative DNA damage based on the comet assay after short- and long-term incubation (3 and 24 h) in HepG2 cells, in a dose- and time-dependent manner. Likewise, Gazzah et al. (2010) demonstrated that 100 μM of ZEA induced a great increase in DNA fragmentation (from 112 ± 2.1 to 460 ± 3.1) in a time-dependent manner (from 2 to 60 h) in HepG2 cells. Similar effects were reported by Venkataramana et al. (2014) in SH-SY5Y cells. These authors showed tail length to increase as the concentration of ZEA increased. Zearalenone induces DNA fragmentation from 31 ± 2.6 (25 μM ZEA) to 83 ± 5.3 (100 μM ZEA); increases ROS generation; and results in a loss of mitochondrial membrane potential, culminating in neuronal cell death. The underlying mechanism is not fully clear, but includes cell apoptosis. On the other hand, little information is available on DNA damage induced by ZEA metabolites in cell cultures. Ayed et al. (2011) found that ZEA and its two major metabolites increased chromosome aberration (CA) in a dose-dependent manner with respect to control HeLa cells. In concordance with our own results, these authors recorded greater genotoxicity with α-ZOL than with β-ZOL. However, in contrast to our data, they observed comparable genotoxicity between ZEA and α-ZOL. Increases in CA were from 18.7 ± 1.5% (15 μM) to 37.7 ± 1.2% (60 μM) for ZEA *versus* untreated cells (7.3 ± 2.18%); from 26.3 ± 2.7% (17 μM) to 43 ± 1.7% (65 μM) for α-ZOL;

and from 17.7 ± 1.2% (25 μM) to 28 ± 2% (100 μM) for β-ZOL in comparison with the control cells (7.3 ± 2.18%). Abid-Essefi et al. (2009) evidenced induction of DNA damage in Caco-2 cells exposed to ZEA, α-ZOL and β-ZOL, depending on their concentrations. However, in contrast to the results obtained in the present study, they found β-ZOL to be more cytotoxic than α-ZOL, while both of these were less cytotoxic than ZEA.

Oxidative stress can be produced by oxidants or ROS. Catalase, SOD and GPx constitute primary intracellular antioxidant defense mechanisms that eliminate oxygen radicals and hydroperoxides which may pose a threat to the cell by causing oxidation. In turn, GSH is a potent nucleophilic scavenger of ROS, converting electrophilic centers to thioether bonds, and acts as a cofactor in the GPx-mediated destruction of hydroperoxides (Hassen et al., 2007; Deponte, 2013; Mallebrera et al., 2014 ; Fernández-Blanco et al., 2014). The depletion of GSH corroborates that cell defense against ZEA and its metabolites is insufficient, resulting in cytotoxic effects that can lead to cell injury. The depletion of GSH levels at high concentrations of ZEA and its metabolites as a response to oxidative damage was observed in CHO-K1 cells. Both ZEA and its metabolites also activate SOD and GPx enzymes. The increase in SOD and GPx scavenger enzyme activities demonstrates an adaptive response to counteract exposure to ZEA and its metabolites in CHO-K1 cells. However, CAT, which can be inactivated by superoxide anion, did not show optimal antioxidant effectiveness. Therefore, it could be assumed that ZEA and its metabolites produce superoxide anion which in turn blocks CAT activity. However, this effect should be evidenced in future studies.

The results obtained in the present work are similar to those obtained by other authors in which ZEA induced time- and concentration-dependent

depletion of GSH. Hassen et al. (2007) found GSH levels to decrease after exposure to 10–200 μM of ZEA in HepG2 cells from 33% (after 3 h incubation) to 53% (after 24 h incubation). Exposure to ZEA (25–200 μM) significantly reduced the GSH levels (approximately 71% of the control level) in CCL 13 cells (Lee et al., 2013 ; Kang et al., 2013).

Lu et al. (2013) exposed REW264.7 macrophages to 50 μM of α-ZOL or β-ZOL simultaneously with CAT and SOD enzymes, and examined whether or not these enzymes protect cells from oxidative damage by inhibiting ZEA metabolite-induced ROS generation. The results obtained by these authors are in contrast to our own findings, since they observed that CAT prevented both α-ZOL- and β-ZOL-mediated reduction of cell viability. The efficacy of CAT in protecting cells was greater in cells exposed to α-ZOL than in cells exposed to β-ZOL. Contrarily, SOD did not reduce the intracellular ROS levels generated by these metabolites.

In conclusion, it has been shown that ZEA, α-ZOL and β-ZOL produce oxidative stress by increasing the levels of ROS, which can cause damage to DNA. The enzymatic scavengers SOD and GPx, and intracellular GSH, are involved in antioxidant defense against these mycotoxins in CHO-K1 cells. However, the changes in the antioxidant defense system do not suffice to neutralize ROS. Thus, the imbalance produced between ROS generation and antioxidant defense mechanisms results in the accumulation of ROS, which can cause DNA damage and lead to cell death. Nevertheless, cells undergoing apoptosis continue to have metabolic activity, and thus count as living cells. Further studies are therefore needed to determine the DNA damage produced by these mycotoxins, and to identify the reasons why such damage occurs.

In sum, the data obtained in this study indicate that naturally contained ZEA and its metabolites are toxic to CHO-K1 cells, and are able to induce oxidative stress.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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3.4 Oxidative damage and disturbance of antioxidant capacity by zearalenone and its metabolites in human cells

Toxicology in vitro (2017)

Oxidative damage and disturbance of antioxidant capacity by zearalenone and its metabolites in human cells

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Abstract

Mycotoxin contamination of foods and feeds represent a serious problem worldwide. Zearalenone (ZEA) is a secondary metabolite produced by *Fusarium* species. This study explores oxidative cellular damage and intracellular defense mechanisms (enzymatic and non-enzymatic) in the hepatoma cell line HepG2 after exposure to ZEA and its metabolites (α-zearalenol, α-ZOL; β-zearalenol, β-ZOL). Our results demonstrated that HepG2 cells exposed to ZEA, α-ZOL or β-ZOL at different concentrations (0, 6.25, 12.5 and 25 μM) showed: (i) elevated ROS levels (1.5- to 7-fold) based on the formation of the highly fluorescent 2',7'-dichlorofluorescein (DCF), (ii) increased DNA damage measured by the comet assay (9-45% higher), (iii) decreased GSH levels and CAT activity (decreased by 54%-25% and by 62%-25% for GSH and CAT, respectively) and (iv) GPx and SOD activities (increased by 50%-90% and by 26%-70%, respectively),

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compared to untreated cells. Our results suggest that mycotoxin-induced oxidative stress and damage may play a major role in the cytotoxic effects of ZEA and its metabolites. GSH and endogenous enzymes function together in protecting cells from ROS and the consequent damage after mycotoxin exposure. ZEA has a lower capacity to induce oxidative stress and damage in HepG2 cells than its metabolites at the tested concentrations.

Keywords: Zearalenone, reactive oxygen species, DNA damage, glutathione, enzymatic activity.

1. Introduction

Mycotoxins are biologically active products produced as secondary metabolites by certain fungal species. Fungal invasion of field crops and mycotoxin contamination of foods and feeds represent a serious problem worldwide. *Fusarium* is one of the main genus implicated in producing mycotoxins. It is known that different fungal species may produce the same mycotoxin and one species may produce different mycotoxins at the same time. Thus, field crops and derived processed food can be simultaneously contaminated with several mycotoxins and may enter the food chain, thus adversely affecting the health of both animals and humans (EFSA, 2011; Fernández-Blanco et al. 2014; Zinedine et al., 2007).

Zearalenone (ZEA) is a resorcylic acid lactone derivate produced by *Fusarium* fungi. The ZEA and its derivatives (α-zearalenol [α-ZOL]; β-zearalenol [β-ZOL]) have structural analogy to estrogen. The estrogenic activity of ZEA and its metabolites has been determined both *in vivo* and *in vitro* (Minervini et al., 2005; Parveen et al 2009; Frizzell et al 2011; Busk et al., 2012; Cortinovis et al., 2013). However, the toxicity by ZEA and its

metabolites is not only due to the previously mentioned estrogenic effect, but other mechanisms such as oxidative stress and damage induced by these compounds may be important mediators involved in their toxicity (Wu et al., 2014). Previous studies have reported that mycotoxins exposure may lead to the production of reactive oxygen species (ROS), which can result in oxidative stress (Hassen et al., 2007; Prosperini et al., 2013; Fenandez-Blanco et al., 2014). Oxidative stress is a state of imbalance between the antioxidant defense and ROS or radical production, so that an excess of ROS can cause oxidative damage to membrane lipids (lipid peroxidation, LPO), proteins, and DNA, which ultimately may lead tocell death (Dinu et al., 2011; Mallebrera et al., 2016; Tatay et al., 2016). Oxidative stress can result in an up-regulation of the antioxidant defense - i.e. enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the antioxidant cellular component glutathione (GSH) - as a protective response to maintain cellular viability (Fernández-Blanco et al. 2014; Mallebrera et al., 2014).

Often more than one mycotoxin is found on a contaminated substrate (food and feed). Exposure to mycotoxins in humans and animals is mostly via ingestion of contaminated foods (Bennett and Klich, 2003). ZEA is rapidly absorbed after oral exposure (Minervini and Dell'Aquila, 2008; Pfeiffer et al. 2011) and it is metabolized to its reduced analogues (α-ZOL and β-ZOL) mainly via hepatic metabolism. The liver is the primary target organ (Bennett and Klich, 2003). Therefore, people can be exposed to these compounds by eating ZEA-contaminated food through a basic diet or food containing *Fusarium* fungi.

The main aim of the present study was to explore the cytotoxicity of ZEA and its major metabolites, α-ZOL and β-ZOL, with regards to oxidative stress in human hepatoma cells (HepG2 cells). For this purpose, HepG2

cells were exposed to ZEA, α-ZOL or β-ZOL standards at different concentrations (0, 6.25, 12.5 and 25 μM). Cell viability, ROS generation, DNA damage and a variety of antioxidants involved in protection against ROS and oxidative damage (i.e. GSH concentrations and GPx, SOD and CAT activities) were measured.

2. Materials and methods

2.1 Reagents and equipment

Dulbecco's Modified Eagle's Medium (DMEM), antibiotics (penicillin and streptomycin), methanol, HEPES, non-essential amino acids (NEAAs), phosphate buffered saline (PBS), glutamine, pyruvate, glucose, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), triton X-100, 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA), propidium iodide (PI), Na-EDTA, agarose, and low melting point (LMP) agarose were provided by Sigma Chemical Co. (St Louis, MO, USA). The mycotoxin standards ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Deionized water (resistivity <18 MΩ cm) was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). Stock solutions of mycotoxins were prepared in methanol and maintained at -20°C in darkness. The final concentrations tested were obtained by adding the culture medium with mycotoxins, and the final solvent concentration in medium was ≤ 1% (v/v).

2.2 Cell and culture conditions

The HepG2 (ATCC-HB-8086) cell line obtained from the American Type Culture Collection was used between passages 50 and 90. The HepG2 cells were grown in polystyrene tissue culture flasks at pH 7.4, 37ºC, 5%

CO_2 and 95% relative humidity. The cells were grown in DMEM medium supplemented with 25 mM HEPES buffer, 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

The concentrations of ZEA and its metabolites were selected considering the previous data obtained in our laboratory in HepG2 cells. The inhibitory concentration 50% (IC_{50}) obtained after ZEA, α-ZOL and β-ZOL exposure during 24 h by the tetrazolium salt (MTT) assay was >100, 27 ± 4 and >100 μM, respectively (Tatay et al., 2014). Thus, the ZEA, α-ZOL and β-ZOL concentrations selected in this study (6.25, 12.5 and 25 μM) were below the IC_{50} obtained.

2.3 Intracellular reactive oxygen species by H_2-DCFDA

Intracellular ROS production was monitored in HepG2 cells by adding H_2-DCFDA. This method is exceptionally sensitive and provides a direct measure of overall oxidative stress, with the detection of intracellular oxidants. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is taken up by the cells and then deacetylated by intracellular esterases, and the resulting 2',7'-dichlorofluorescein (DCFH) becomes trapped inside the cell. The non-fluorescent DCFH is switched to highly fluorescent dichlorofluorescein (DCF) when oxidized by ROS. The method was developed according to Ruiz-Leal and George (2004). Briefly, 2×10^4 cells/well were seeded on a 96-well black microplate. After 24 h, the medium was removed and 20 μM H_2-DCFDA solution was added to each well. The cells were incubated for 20 min before removal of the supernatant, washed twice with PBS and 200 μl/well of medium, 1% MeOH (control), and medium with ZEA or its metabolites (6.25, 12.5 and 25 μM)

was added. The increase in fluorescence was measured at intervals for up to 120 min at excitation and emission wavelengths of 485 and 535 nm, respectively. Eight replicates per concentration were developed. Results were expressed as the increase in fluorescence of the mycotoxin compared with solvent control. Three independent experiments for were performed.

2.4 Alkaline comet assay

The alkaline comet assay was performed to determine the DNA strand breaks as described by Mallebrera et al. (2016). When an electric field is applied, intact DNA strands remain in the head, while the broken pieces of DNA migrate towards the anode forming a typical comet tail. Briefly, 3.4 x 10^5 cells were seeded in each well using 6-well plates. After confluence, cells were treated with ZEA or its metabolites at different concentrations (0, 6.25, 12.5 and 25 μM) for 24 h. Then, HepG2 cells were embedded in 0.8% LMP agarose, transferred to slides and lysed. The slides were then immersed in an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 40 min at room temperature. The electrophoresis was run in the same solution at 0.7 V/cm (voltage across the platform) and 300 mA for 24 min. After electrophoresis, the slides were washed twice in neutralization buffer, dried in 96% ethanol and stained with 20 mg/mL propidium iodide (PI). The analysis was performed with a fluorescence microscope (NIKON Eclipse E800), equipped with camera (NIKON DXM1200F). Fifty cells/slide were processed by Comet-Score (Automatic Comet Assay), http://autocomet.com/index.php?id=cometscorepro. Results are expressed as the percentage of DNA in tail (%), calculated according to the equation: % DNA in tail = (total intensity of tail/total intensity of comet) x 100. Total intensity of comet = head length + tail length. Determinations were performed in three independent experiments.

2.5 Determination of GSH

For determining the GSH levels and GPx, SOD and CAT activities in HepG2 cells exposed to ZEA or its metabolites, 3 x 10^5 cells/well were seeded in 6-well culture plates. When cells achieved 65% confluence, the medium was removed and 200 µl of medium with ZEA, α-ZOL or β-ZOL (6.25, 12.5 and 25 µM) were added. Following the 24 h- treatment, the medium was removed and cells were washed twice with PBS. The cells were homogenized in 0.5 ml of 20 mM Tris and 0.1% Triton.

Determination of reduced GSH was made by adapting the method described by Maran et al. (2009). Briefly, 10 µl of each homogenized cell sample was placed in a 96-well black plate with 200 µl of GSH buffer (0.1 M Na_2HPO_4-0.005 M EDTA, pH 8.0) and 10 µl of o-phthaldialdehyde (OPT) solution, mixed and incubated in darkness at room temperature for 15 min. The concentration of GSH was determined using a microplate reader (Wallace Victor 2, 1420 Multilabel Counter, Perkin Elmer, Turku, Finland) at an excitation and emission wavelength of 345 nm and 425 nm, respectively. The GSH levels were expressed as µg GSH/mg protein. Determinations were performed in triplicate.

2.6 Determination of enzyme activities

GPx activity was assayed spectrophotometrically using H_2O_2 as substrate for Se-dependent peroxidase activity of GPx by following oxidation of NADPH at 340 nm during the first 2 min in a coupled enzymatic reaction with GR, as described by Maran et al. (2009). In 1-ml final volume, the reaction mixture contained 500 µl of 0.1 M phosphate buffer (pH 7.5, 1 mM EDTA, 2 mM NaN_3 and 0.1% Triton X-100), 250 µl of ultrapure water, 100 µl of 20 mM GSH, 20 µl of 0.2 mM NADPH, 2.5 U of freshly prepared

GR and 50 µl of 5 mM H_2O_2). Fifty µl of homogenized cell samples were added to the reaction mixture. One unit of GPx will reduce 1 µmol of GSSG per min at pH 7.5. Assays were conducted at 25°C during 2 min with a spectrometer (Perkin Elmer UV/Vis Lambda 2 version 5.1). GPx activity was calculated by using the molar absorptivity of NADPH (6.22 mM^{-1} cm^{-1}) and expressed as nmol NADPH oxidized/min/mg of protein.

SOD activity was determined with the Ransod kit (Randox Laboratories, United Kingdom) adapted for 1.5 ml cuvettes. SOD activity was monitored at 505 nm during 3 min at 37ºC with a spectrometer (Perkin Elmer UV/Vis Lambda 2 version 5.1). SOD results were expressed as units of SOD per mg protein.

CAT activity was measured according to Espín et al. (2014) with slight modifications. Briefly, 50 µl of homogenized cell suspension was mixed with 950 µl of 0.05 M NaH_2PO_4 and 500 µl of 0.03 M H_2O_2. The rate of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240 nm for 2 min with a spectrophotometer (Super Aquarius CECIL CE 9500). CAT activity was calculated by using the molar absorptivity of H_2O_2 (43.6 mM^{-1} cm^{-1}) and expressed as µmol H_2O_2/min/mg of protein. All the enzyme determinations were performed in triplicate.

2.7 Determination of total protein content

Cellular protein content was assayed using the Bio-Rad DC Protein Assay; catalog number 500-0116. Protein concentration was measured at 690 nm.

2.8 Statistical analyses

The statistical analysis of the data was carried out using the SPSS version 19 statistical package (SPSS, Chicago, IL, USA). All values are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's HDS test for *post hoc* pairwise comparisons. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Intracellular ROS production

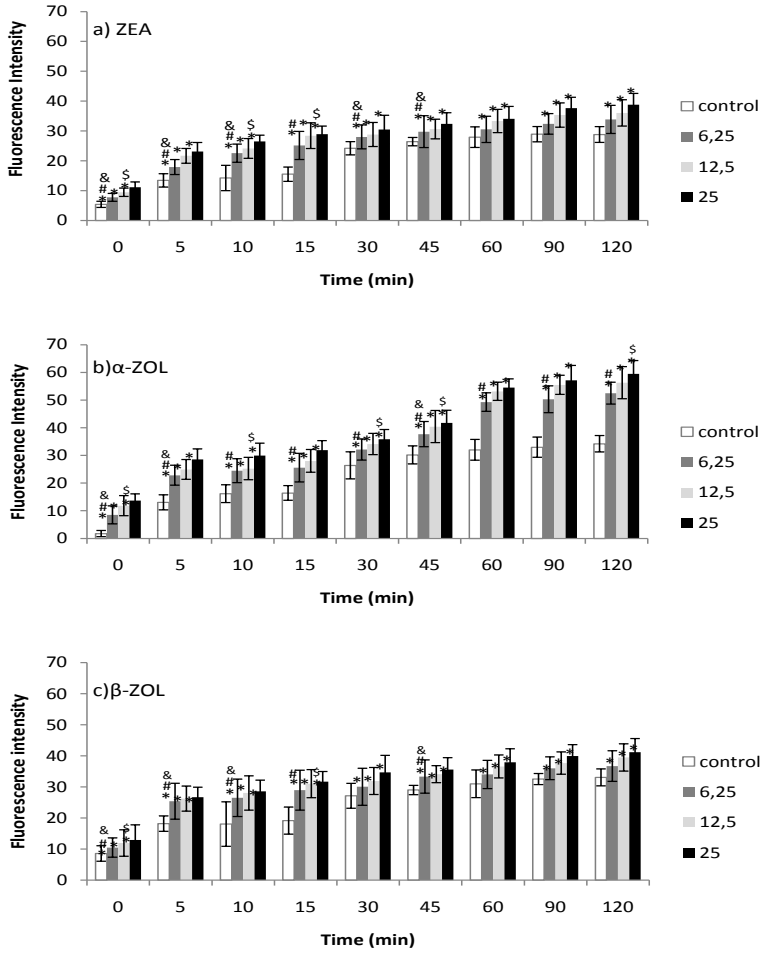
A previous study carried out on HepG2 cells demonstrated that the IC_{50} values obtained after ZEA, α-ZOL and β-ZOL exposure during 24 h by the MTT assay were >100, 27 ± 4 and >100 μM, respectively (Tatay et al., 2014).

In order to determine changes in redox status, HepG2 cells were exposed to different concentrations of ZEA, α-ZOL or β-ZOL (0, 6.25, 12.5 and 25 μM) in three different experiments for 120 min. The results obtained showed that HepG2 cells treated with ZEA and its metabolites increased the concentration of oxidizing species compared to the basal levels (Fig. 23). The highest fluorescence intensity in cells was observed at the lower exposure times. The ROS production detected at 0 min was 2-, 7- and 2.5-fold higher in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL than in control cells, respectively (Fig. 23).

Significant differences in ROS production between the different treatment groups (6.25, 12.5 and 25 μM) for each mycotoxins were also observed (Fig. 23). Significant differences between 6.25 *vs.* 12.5 μM and between 6.25 *vs.* 25 μM were observed for ZEA and β-ZOL from 0 to 45

min exposure. Significant differences between 12.5 *vs.* 25 μM were also observed for these two mycotoxins from 0 to 15 min exposure (Fig. 23). However, α-ZOL showed significant differences between 6.25 *vs.* 12.5 μM, 12.5 *vs.* 25 μM, and between 6.25 *vs.* 25 μM at all times of exposure tested (0-120 min) (Fig. 23).

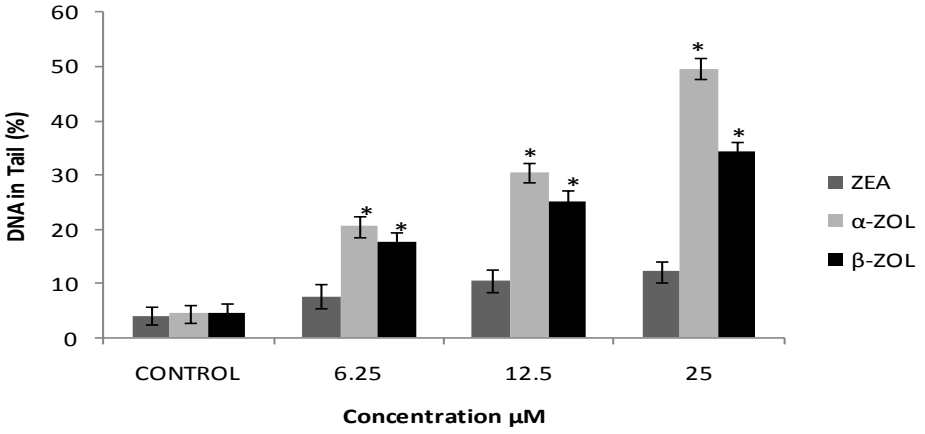
Figure 23. ROS-induced fluorescence in HepG2 cells exposed to ZEA (a), α-ZOL (b) and β-ZOL (c) for 120 min. HepG2 cells were exposed to H_2-DCFDA for 20 min before ZEA, α-ZOL and β-ZOL exposure. Results are expressed as mean ± SEM (n=3). (*)significant differences ($p \leq 0.05$) *versus* control. (&)significant differences between 6.25 and 12.5 µM treatment groups. (#)significant differences between 6.25 and 25 µM treatment groups. ($)significant differences between 12.5 and 25 µM treatment groups.



3.2 DNA damage by alkaline comet assay

Figure 2 shows the DNA strand breaks induced by ZEA, α-ZOL and β-ZOL (0-25 μM) in HepG2 cells. ZEA metabolites increased DNA damage in a dose-dependent manner. The greatest DNA migration was produced by α-ZOL (Fig. 24). Compared to untreated cells, the DNA damage was 9%, 45% and 30% higher in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL, respectively. Significant differences in DNA migration between 6.25 *vs.* 12.5 μM, 6.25 *vs.* 25 μM and 12.5 *vs.* 25 μM treatment groups were observed for α-ZOL (Fig. 24); DNA damage increased by ca. 42%, 114% and 50%, respectively. Significant differences between 6.25 *vs.* 12.5 μM and between 6.5 *vs.* 25 μM were also found for β-ZOL (DNA damage increased by ca. 27% and 94%, respectively) (Fig. 24).

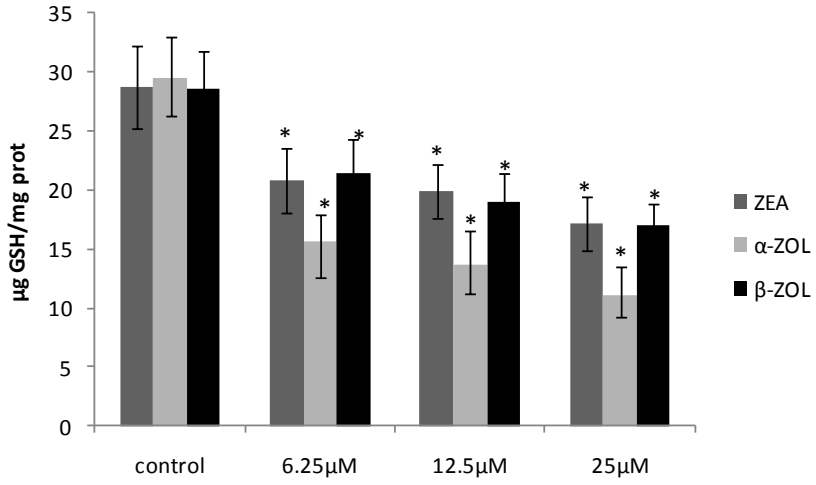
Figure 24. Percentage (%) of DNA in tail in HepG2 cells exposed to 6.25, 12.5 and 25 μM ZEA, α-ZOL and β-ZOL for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 μM treatment groups. (#) significant differences between 6.25 and 25 μM treatment groups. ($) significant differences between 12.5 and 25 μM treatment groups.



3.3 GSH levels

The HepG2 cells were exposed to 6.25, 12.5 and 25 μM ZEA, α-ZOL or β-ZOL for 24 h, and the alterations in GSH content were explored (Fig. 25). In HepG2 cells, the GSH levels (μg GSH/mg protein) decreased from 40% to 28%, from 62% to 46%, and from 40% to 25% for ZEA, α-ZOL and β-ZOL, respectively (Fig. 25). Significant differences in GSH concentrations (decreased by 20%) between 6.25 *vs.* 25 μM treatment group were observed for β-ZOL (Fig. 25).

Figure 25. GSH levels (μg/mg protein) in HepG2 cells exposed to ZEA, α-ZOL or β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3) (*) significant differences ($p \leq 0.05$) *versus* control. (#) significant differences between 6.25 and 25 μM treatment groups.

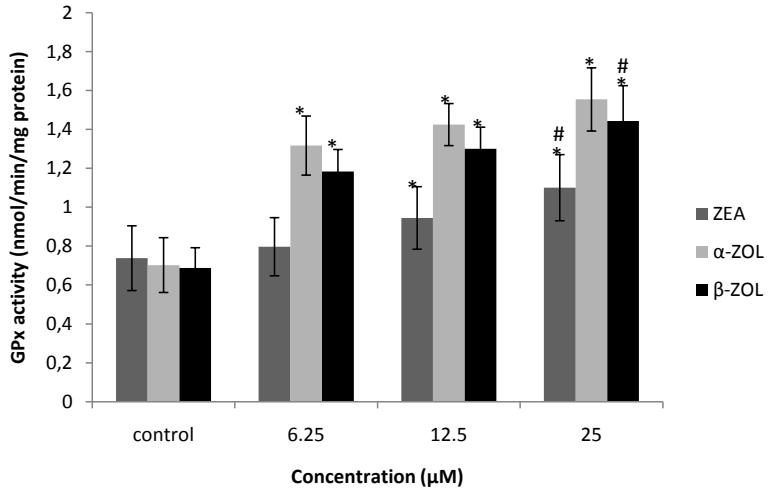


3.4 Determination of enzymatic activities

GPx, CAT and SOD activities in HepG2 cells exposed to ZEA or its metabolites at different concentrations for 24 h are shown in Figures 26-28. GPx activity increased significantly when HepG2 cells were exposed to

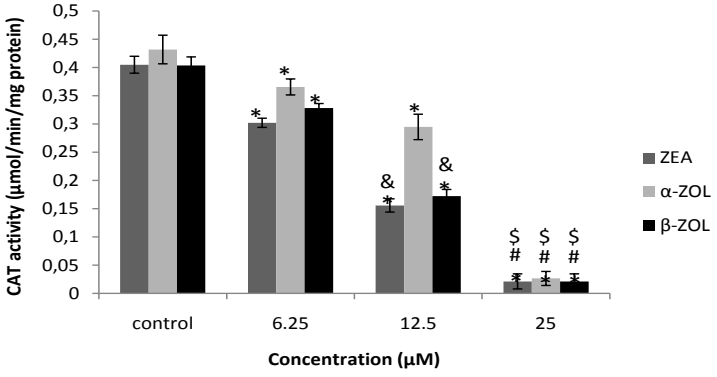
ZEA, α-ZOL and β-ZOL compared to control cells (Fig. 26). The greatest increase was observed in cells exposed to these compounds at a concentration of 25 μM. Overall, the GPx activity increased up to 50%, 95% and 90% in cells exposed to ZEA, α-ZOL and β-ZOL, respectively. Moreover, significant differences in GPx activity were observed between 6.25 and 25 μM ZEA-treated groups (increased by ca. 30%) and between 6.25 and 25 μM β-ZOL-treated groups (increased by ca. 25%).

Figure 26. GPx activity (nmol/min/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) versus control. (#) significant differences between 6.25 and 25 μM treatment groups.



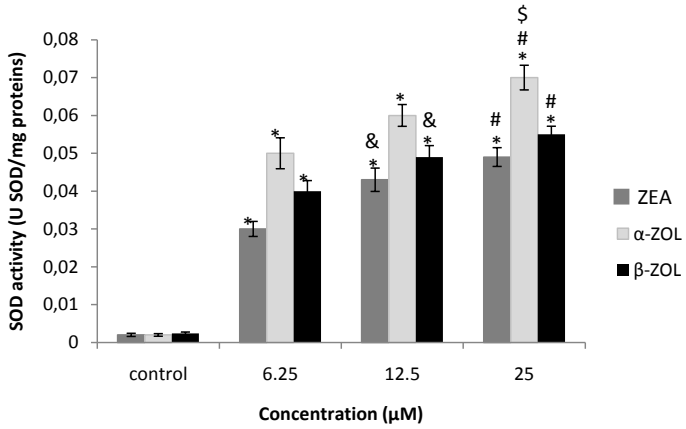
CAT activity decreased in a dose-dependent manner from 95% to 25% in HepG2 cells exposed to ZEA, and from 95% to 15% for α-ZOL and β-ZOL. Significant differences in CAT activity between 6.25 *vs.* 12.5 μM, 6.25 *vs.* 25 μM, and 12.5 *vs.* 25μM groups were observed in cells exposed to ZEA and β-ZOL; CAT activity decreased by ca. 40%, 70% and 35% for these two mycotoxins, respectively. CAT activity did not differ between 6.25 *vs.* 12.5 μM groups in α-ZOL-treated cells. However, significant differences in CAT activity between 6.25 *vs.* 25 μM treatment groups (decreased by ca. 80%) and 12.5 *vs.* 25 μM treatment groups (decreased by ca. 60%) were observed (Fig. 27).

Figure 27. CAT activity (μmol/min/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 μM treatment groups. (#) significant differences between 6.25 and 25 μM treatment groups. ($) significant differences between 12.5 and 25 μM treatment groups.



Mycotoxin treatment enhanced the SOD activity in HepG2 cells to values 1500- to 2100- fold higher (ZEA and β-ZOL-treated cells) or 2400- to 3400-fold higher (α-ZOL-treated cells) than those found in control cells. No significant differences in SOD activity were observed between 6.25 *vs.* 12.5 μM groups for α-ZOL-treated cells. However, significant differences in SOD activity were found between 6.25 *vs.* 25 μM treatment groups (increased by ca. 40%) and 12.5 *vs.* 25 μM treatment groups (increased by ca. 15%) for α-ZOL-treated cells. We also observed differences in SOD activity between 6.25 *vs.* 12.5 μM and 6.25 *vs.* 25 μM treatment groups for ZEA and β-ZOL-treated cells; SOD activity increased by 40% and 60% for ZEA-treated cells, respectively, and by 20% and 35% for β-ZOL-treated cells, respectively (Fig. 28).

Figure 28. SOD activity (U/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 μM treatment groups. (#) significant differences between 6.25 and 25 μM treatment groups. ($) significant differences between 12.5 and 25 μM treatment groups.



4. Discussion

Small amounts of ROS are constantly generated within cells as a result of normal physiological processes. Toxic substances in the diet or environmental contaminants, when absorbed by mammalian cells, are also able to produce ROS. Overproduction of ROS can alter membrane permeability and structure and induce DNA mutations (Guo et al., 2013). Mitochondria contain their own DNA, which is particularly susceptible to ROS attack associated with oxidative stress (Guo et al., 2013). Acute ROS exposure can result in shut-down of mitochondrial energy production (Prosperini et al., 2013). In our study ZEA and its isomers induced oxidative

stress through ROS generation in HepG2 cells (Fig. 1). ZEA and β-ZOL showed less ROS production than α-ZOL. Moreover, ZEA and β-ZOL induced ROS production in a dose-dependent manner during the first 45 min of exposure, whereas HepG2 cells exposed to α-ZOL showed significant ROS production in a dose-dependent manner during the entire exposure time (120 min). Taken together, these results suggest that α-ZOL may be the major contributor to the total ROS production. According to this, previous studies have reported increased ROS production in different cells exposed to ZEA at different concentrations (HepG2 cells, 100 μM ZEA, El Golli-Bennour et al., 2008; CHO-K1 cells, 1-50 μM ZEA, Ferrer et al., 2009; porcine granulosa cells, 15-60 μM ZEA, Qin et al., 2015; L02 liver cells, 10-40 μM ZEA, Wu et al., 2014). These authors also found that the production of ROS is time dependent (El Golli-Bennour et al., 2008; Ferrer et al., 2009). In this sense, Ferrer et al (2009) reported that ZEA-induced ROS generation is higher during the early stages of exposure (4-fold higher than controls), whereas its capacity to generate ROS decreases after 5 min (2-fold increase) when cells are exposed to 1, 5 and 50 μM ZEA.

The increased ROS generation in cells exposed to ZEA and its metabolites could consequently contribute to metabolic oxidative stress, genomic instability and cellular injury. Chronic exposure to ROS can result in oxidative damage to DNA and RNA polymerase, histones, topoisomerase-II and other DNA-associated proteins. Thus, ROS generation can lead to DNA strand breaks and chromosome damage. Our results showed that ZEA and its metabolites increase DNA damage in HepG2 cells. Cells exposed to α-ZOL showed a higher increase in DNA damage compared to cells exposed to ZEA or β-ZOL at all concentrations tested. ZEA showed the lowest capacity to induce DNA damage, showing similar results for all concentrations tested, whereas α-ZOL and β-ZOL

increased DNA strand breaks in a dose-dependent manner. These findings are consistent with those obtained by other authors using different types of cells (Vero cells, 10-40 μM ZEA, Ayed-Boussema et al., 2007; Caco-2 cells, $IC_{50}/2$, IC_{50} and 2 x IC_{50}) ZEA, α-ZOL and β-ZOL, Abid-Essefi et al., 2009; HepG2 cells, 100 μM ZEA, Gazzah et al., 2010; CCL13 liver cells, 25-200 μM ZEA, Kang et al., 2013; human neuroblastoma SH-SY5Y cells, 25-100 μM ZEA, Venkataramana et al., 2014). Therefore, our study supports that overproduction of ROS could contribute to DNA chain breakdown and cell damage. Our results suggest that α-ZOL may have a higher capacity to produce DNA damage than ZEA or β-ZOL.

Under aerobic living conditions, oxidative processes and the subsequent generation of ROS are normal in the cellular metabolism. Living beings are equipped with an antioxidant defense system able to inhibit ROS generation and reduce oxidation and the consequent damage. When the balance between the antioxidant defense and ROS production is disrupted, the cells try to survive by degrading their own protein aggregates or organelles, with the initiation of apoptosis (Qin et al., 2015). Particularly, cellular antioxidant enzymes play a major role in protecting cells from oxidative stress and damage. They are effective in scavenging ROS since they catalyze the breakdown of free radicals and support the antioxidant defense system by catalyzing the conjugation of toxic compounds with GSH (SOD, CAT, GPx, GR, GST) (Halliwell and Gutteridge, 1999). Regarding the non-enzymatic antioxidant defense system, the tripeptide GSH is one of the most abundant sulfhydryl-containing groups in most organisms and plays a basic role in binding with ROS. These antioxidants operate in association with each other forming an integrated antioxidant defense system (Halliwell and Gutteridge, 1999). ZEA, α-ZOL and β-ZOL

have toxicological interest because of their potential to cause oxidative stress and damage.

In this study, HepG2 cells exposed to ZEA, α-ZOL and β-ZOL showed and up-regulation of the antioxidant defense system by increasing the activity of GPx and SOD as a possible protective response, while a reduction in CAT activity and GSH levels was found.

SOD enzyme catalyses the transformation of the superoxide anion radical into H_2O and O_2, while GPx enzyme reduces peroxides in cells, such as the transformation of H_2O_2 to H_2O by using GSH as a substrate (Halliwell and Gutteridge, 1999). While all the concentrations tested enhanced GPx activity to a similar level (only cells exposed to 25 μM ZEA or β-ZOL showed significantly higher GPx activity than cells exposed to 6.25 μM), the different treatments induced SOD activity in a dose-dependent manner. In our study, HepG2 cells exposed to ZEA and its metabolites increased the concentration of oxidizing species compared to the untreated cells. Thus, the increased GPx and SOD activities reported in our study can be a compensatory mechanism to scavenge ROS levels produced as a result of ZEA, α-ZOL and β-ZOL exposure. Our study shows that both enzymes play a major role in providing protection against damage induced by ZEA and its metabolites in HepG2 cells.

On the other hand, the reduction of GSH concentrations in HepG2 cells exposed to ZEA and its metabolites could be related to its requirement for conjugation reactions of detoxification. In this sense, since GPx oxidizes GSH to reduce H_2O_2 (Halliwell and Gutteridge, 1999), the increased GPx activity due to mycotoxin exposure can promote a reduction in GSH levels. Both CAT and GPx catalyze the decomposition of H_2O_2, but CAT directly catalyses the transformation of H_2O_2 to H_2O and O_2 (Halliwell and Gutteridge, 1999). CAT enzymes are abundant in the peroxisomes of liver

cells, while GPx is abundant in mitochondria and cytosol compartment. In our study, CAT activity significantly decreased in a dose-dependent manner in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL. This marked effect can be due to high concentrations of H_2O_2 produced under mycotoxin exposure. In situations of high peroxide concentrations, a depression of CAT activity may occur, and it can be even inactivated. Moreover, an oxidation of the CAT enzyme by the excess of peroxide is also possible (Williams, 1928).

In general, the highest GPx and SOD activities and the lowest GSH concentrations were reached in cells exposed to α-ZOL. In addition, cells exposed to this metabolite and to β-ZOL showed higher ROS production and DNA damage levels compared to cells exposed to ZEA. These results suggest that α-ZOL and β-ZOL have a higher capacity to induce oxidative stress and damage in HepG2 cells at the tested concentrations.

Previous studies have found similar effects in different cell types exposed to ZEA or other *Fusarium* mycotoxins. However, to the best of our knowledge, there is no data available on the oxidative effects caused by α-ZOL and β-ZOL exposure. Lee et al. (2013) and Hassen et al. (2007) observed that ZEA (200 μM) decreased GSH levels in HepG2 cells (decreased by 71% and 33% compared to the control, respectively), while Qin et al. (2015) observed a decrease in CAT activity when porcine granulosa cells were exposed to ZEA (3-60 μM) for 24 h. Similar results were obtained by other authors, reporting reduced GSH levels and/or increased GPx, SOD and CAT activities in different cells (CHO-K1, Caco-2, PK15, U937, HeK-293 cells) exposed to different mycotoxins (beauvericin, BEA; fumonisin B1, FB1; deoxynivalenol, DON) (Mallebrera et al., 2014; Prosperini et al., 2013; Klaric et al., 2007; Costa et al., 2009; Dinu et al., 2011).

Based on our results, it can be concluded that mycotoxin-induced oxidative stress and damage may play a major role in the cytotoxic effects of ZEA and its metabolites. ZEA and its metabolites enhance ROS generation and DNA damage in HepG2 cell in a dose-dependent manner. HepG2 cells exposed to ZEA, α-ZOL and β-ZOL showed and up-regulation of the antioxidant defense system by increasing the activity of GPx and SOD, suggesting that these enzymes play a major role in providing protection against damage induced by these mycotoxins. In addition, a reduction in CAT activity and GSH levels was found, which could be related to the requirement of these antioxidants for H_2O_2 reduction and for conjugation reactions of detoxification, respectively. Our results suggest that ZEA has a lower capacity to induce oxidative stress and damage in HepG2 cells at the tested concentrations than its metabolites.

Acknowledgements

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3.5 Estrogenic activity of zearalenone, α-zearalenol and β-Zearalenol assessed using E-screen test in MCF-7 cells

Estrogenic activity of zearalenone, α-zearalenol and β-Zearalenol assessed using E-screen test in MCF-7 cells

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Abstract

Mycotoxins, including zearalenone (ZEA) can occur worldwide in cereals. They can enter the food chain and cause several health disorders. ZEA and its derivatives (α-zearalenol, α-ZOL and β-zearalenol, β-ZOL) have structural analogy to estrogen, thus they can bind to estrogen receptors (ERs). In order to characterize the estrogenic activity of ZEA, α-ZOL and β-ZOL, the proliferation of ER-positive human breast cancer (MCF-7) cells was measured. After exposure at levels ranging from 6.25 to 100 μM, cell proliferation (E-screen assay) was evaluated by sulforhodamine B (SRB) through estrogenic parameters. Our results demonstrated that ZEA, α-ZOL and β-ZOL induce estrogenic-like effects by the E-screen assay in MCF-7 cells. All mycotoxins showed estrogenic activity. This effect may be due because ZEA and its metabolites are flexible enough to be able to bind to mammalian estrogen receptors (ER). The relative proliferative effect (RPE%) ranged from 10% to 91%. The α-ZOL induced higher proliferative

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effect. The highest RPE% of α-ZOL is due to a higher affinity than the other mycotoxins to the ER.

Keywords: Zearalenone, Zearalenol, estrogenic activity, E-Screen.

1. Introduction

Certain chemical compounds may mimic or antagonize the action *in vitro* or *in vivo* of natural estrogens such as 17β-estradiol (E2). These substances are defined as substances with estrogenic activity or substances with antiestrogenic activity (Bittner et al. 2014). Many of these substances have little obvious structural similarity to natural estrogen and are able to bind to endocrine receptors (ERs), influence the expression of estrogen-regulated genes, regulate the growth of estrogen-dependent cells and produce physiological estrogen responses *in vivo* (Darbre et al., 2002).

These substances cause several health disorders because they can interfere with the normal functioning of endocrine processes and therefore may interfere with hormonal function in humans producing many problems such as early puberty in females, reduction of spermatozoa, alteration of the functions of organs Reproductive, obesity, altered sexual behaviors, and may increase some breast, ovarian, testis and prostate cancers (Bittner et al., 2014).

Zearalenone (ZEA) is a resorcylic acid lactone derivate produced by *Fusarium* fungi. The ZEA and its derivatives (α-zearalenol [α-ZOL]; β-zearalenol [β-ZOL]) have structural analogy to estrogen. The estrogenic activity of ZEA and its metabolites has been determined both *in vivo* and *in vitro* (Le Guevel and Pakdel, 2001; Minervini et al., 2005; Caloni et al., 2009; Parveen et al 2009; Frizzell et al 2011; Busk et al., 2011, 2012; Prouillac et al., 2012; Cortinovis et al., 2013). Their structures are flexible

enough to bind to mammalian ERs (Parveen et al., 2009). Thus, they are able to mimic the activity of naturally occurring estrogens (Gajecka, 2012). It is known that these mycotoxins induce their toxicity by competitive binding to the ER and modifying steroid metabolism and producing morphological and functional changes in the reproductive system (Salem et al. 2017). It has been shown, that ZEA decrease fertility, due to reproductive tract disorders and abnormal fetal development reduce the size and weight of the adrenal and pituitary glands in animals, and alter the ovulation cycle (Parveen et al., 2009; Cortinovis et al., 2013; EFSA, 2016). Due to the anabolic activity of ZEA, it is used as a hormonal growth-promoter in food animals in the United States and Canada, whereas any use of ZEA as a growth promoter is forbidden in the EU (Le Guevel and Pakdel, 2001).

The aim of the present study was to determine the effect of ZEA, α-ZOL and β-ZOL with regards to the estrogenic activity by the proliferation E-screen bioassay in MCF-7cells.

2. Materials and methods

2.1 Reagents and equipment

Cell cultures and chemical reagents, Dulbecco's Modified Eagle's Medium (DMEM), methanol, 17β-estradiol (E2), non-essential amino acids (NEAAs), glutamine, pyruvate, insulin, sulforhodamine B (SRB), trichloroacetic acid (TCA) and acetic acid were from Sigma Chemical Co. (St Louis, MO, USA). Mycotoxins selected ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol) were purchased by Sigma Chemical Co. (St Louis, MO, USA). Charcoal/dextran treated fetal bovine serum and fetal bovine serum (FBS) were from Cambrex (Belgium).

Deionized water (resistivity <18 MΩ cm) was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). Stock solutions of mycotoxins were prepared in methanol and maintained at -20°C in darkness. Stock solution of E2 (10^{-3}M) was prepared in ethanol and stored at -20ºC in darkness. The final concentrations tested were obtained by adding the culture medium with mycotoxins or E2, and the final solvent concentration in medium was ≤ 1% (v/v).

2.2 Cell and culture conditions

The MCF-7 (ATCC-HTB-22) cell line obtained from the American Type Culture Collection was used between passages 50 and 90. The MCF-7 cells were grown in polystyrene tissue culture flasks at pH 7.4, 37ºC, 5% CO_2 and 95% relative humidity. The MCF-7 cells were maintained in DMEM medium with 15 mg/l phenol red and supplemented with 5% FBS, 1% glutamine, 1% NEAA, 1% pyruvate and 0.1% insulin. Because of the hormonal activity of phenol red and FBS, experiments in MCF-7 cells were performed in a test culture medium phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5%), 1% glutamine, 1% NEAA, 1% pyruvate and 0.1% insulin. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

2.3 E-screen assay

The E-screen assay was carried out according to the method described by Körner et al. (1999) and modified by Schirilò et al. (2009). Briefly, MCF-7 cells were trypsinized and plated in 96-well plates with test medium at a concentration of $2x10^3$ cells/well. Cells were allowed to attach for 24 h, and the seeding medium was removed, washed with PBS and replaced with

test medium containing serial dilutions of ZEA (from 12.5 to 100 μM), α-ZOL and β-ZOL (from 6.5 to 100 μM), E2 as positive control (10^{-9} -10^{-3}M) and negative control (solvent/methanol). The bioassay was ended on day 6. It has been demonstrated that E2 induces maximal proliferation at a concentration of 10^{-9} M (Molina-Molina et al., 2014).

Previous studies in our laboratory indicate that the late exponential phase of MCF-7 cell proliferation in response to E2 concentration is reached after 144 h (6 days). Following the incubation time, the medium was carefully aspirated to avoid cell detachment, and the cells were incubated at 4ºC for 1 h in 10% (w/v) TCA. The TCA was removed and the wells were washed under a gentle stream of tap water and thoroughly air-dried. The TCA-fixed cells were stained with 0.4% (w/v) SRB dissolved in 1% acetic acid. Following incubation, the supernatant was discarded and unbound dye was removed by rinsing the wells with 1% acetic acid. After thorough drying, bound dye was solubilized with 10 mM Tris base, pH 10.5, for 20-30 min. The optical density (OD) was measured at 570 nm with a reference filter at 690 nm using a fluorimeter (Multiskan MCC/340 plate reader, Thermo Fisher Scientific, Marietta, OH, USA). Three independent experiments were performed.

The basic endpoint of the E-screen assay is the cell proliferation relative to the hormone-free control. The proliferative effect (PE) of a sample is the ratio of the maximum OD value that was obtained with each mycotoxin or E2 to that of the negative control: PE = OD (mycotoxins or E2)/OD (negative control)

The estrogenic activity of samples was evaluated by determining the relative proliferative effect (RPE%), which compares the maximum proliferation induced by the mycotoxin to that induced by E2:

$$RPE\% = [(PE\text{-}1)_{mycotoxin}/(PE\text{-}1)_{E2}] \times 100$$

Thus, full agonistic activity (RPE = 100) can distinguished from partial agonistic activity (RPE < 100%).

Mycotoxins can be classified according to the strength of RPE as total agonists (when RPE% ranges from 80% to 100%), partial agonists (between 25% and 80%) or weak agonists (between 10% and 25%). A relative proliferative effect of < 10% was defined as not determinable and considered a negative result

2.4 Statistical analyses

The statistical analysis of the data was carried out using the SPSS version 19 statistical package (SPSS, Chicago, IL, USA). All values are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's HDS *post hoc* for multiple comparisons. A p-value of ≤ 0.05 was considered statistically significant.

3. Results

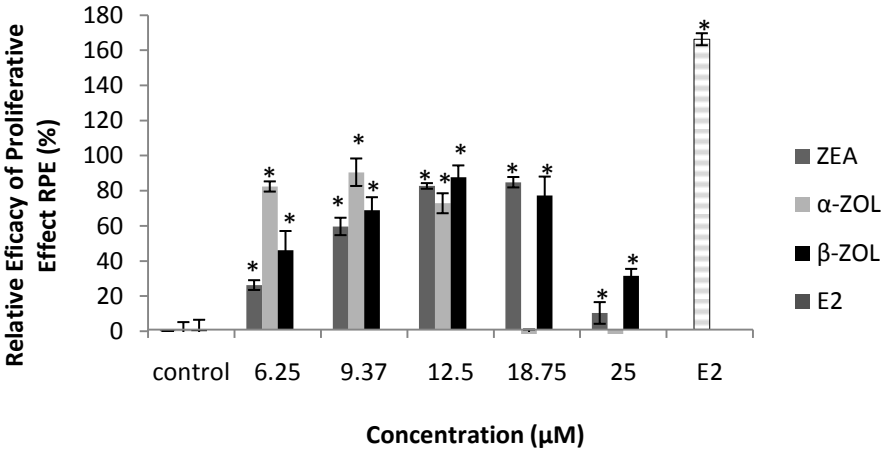
3.1 Estrogenic effects of ZEA and its metabolites by the E-screen bioassay

The E-screen bioassay is based on increasing proliferation of MCF-7 cells caused by the presence of estrogen active substances binding to ERs. This test was used because it can screen a large number of compounds in a short period of time. Moreover, it is one of the most useful screening bioassay for evaluating estrogenic activity, because it integrates the effects of chemicals which may not be measured in targeted analytical screening (Minervini et al., 2005). MCF-7 cells are used for E-screen bioassays because they possess a large number of ERα and ERβ, and since they

exhibit stable estrogen dependency compared to other human cell lines (Minervini et al., 2005; Schiliro et al., 2009). When MCF-7 cells are grown in medium without estrogens, proliferation is prevented, and when estrogens are added, the cells are seen to proliferate (Schirilò et al., 2012).

Figure 29 shows that ZEA and its metabolites produced a significant increase in RPE% at the tested low concentrations *versus* the solvent control. The highest RPE% (91%) was observed at 9.37 μM α-ZOL exposure. Higher concentrations tested of α-ZOL demonstrated saturation in proliferative effect in MCF-7 cells (Figure 29).

Figure 29. The relative proliferative effect (RPE%) of the MFC-7 cells treated with different concentrations of ZEA, α-ZOL and β-ZOL. The concentrations tested for E2 were form 10^{-9} to 10^{-3} M. The concentration shown in this figure is 10^{-8} M. The results are represented as means ± SEM (n=3). (*) $p \leq 0.05$ indicates significant differences *versus* control.



4. Discussion

The E-screen assay is used for the detection and quantification of the estrogenic activity of single chemicals mediated by direct binding to ERs stimulating the growth of an estrogen dependent cell line, commonly MCF-7. In order to evaluate the estrogenic activity produced by ZEA and its metabolites (α-ZOL and β-ZOL), it is necessary to determine the RPE% effect that occurs when these mycotoxins are tested alone in MCF-7 cells. The results obtained show that there is an increase in RPE% values at low concentrations, and α-ZOL estrogenic potency is greater than that of ZEA or β-ZOL. However, for all mycotoxins tested (ZEA, α-ZOL and β-ZOL) the higher concentrations demonstrated saturation in PE in MCF-7 cells, showing a decrease in the %RPE. Moreover, at higher concentrations there is also a toxic effect demonstrated by the IC_{50} values (ZEA >100 μM; α-ZOL=27±4 μM and β-ZOL >100 μM). Thus can be understood that α-ZOL is the one that produces a greater decrease in proliferative effect, even reaching values out of place to the highest concentrations tested. The results obtained in our work are similar to those obtained by other authors (Kuiper et al., 1998; EFSA, 2016).

Shier et al. (2001) classified ZEA and 16 structural analogs according to their proliferative potency in MCF-7 cells. Their data suggest that the functional group at the 6 position has the greatest effect on estrogenicity; α-ZOL (α-OH at the 6 position) demonstrated greater potency that other species in stimulating the growth of MCF-7 cells. According to Parveen et al. (2009), this effect may be due to the fact that ZEA and its metabolites have a flexible structure which is able to bind to ER as strongly as natural estrogen E2. The greater potency of α-ZOL compared to ZEA and β-ZOL reflects its greater affinity for ERs in MCF-7 cells. On the other hand, and in contrast to the hormones, α-ZOL does not bind to the carrier protein -

thereby increasing its ability to produce toxic effects. Moreover, we found the RPE% values to increase at the lower concentrations tested, and no determinable disruption or partial agonism of ZEA and its metabolites was observed.

The estrogenic properties of ZEA and its isomers have also been studied in other cell lines and with different assays (EFSA, 2016). Prouillac et al. (2012) determined the estrogenic potency of ZEA, α-ZOL and β-ZOL on adenosine triphosphate (ARP)-binding cassette (ABC) transporters which are regulated by hormonal influence, i. e. estrogen and progesterone. In concordance with our results, they observed that all mycotoxins induced alterations of ABC transporter expression in human placental BeWo cells at concentration of 0.1 μM. Frizzell et al. (2011) and Ehrlich et al. (2015) used the activation of reporter gene assay (RGA assay) and CALUX assay (an ERα chemical activated luciferase reporter gene assay) to assess the estrogenicity of ZEA and its metabolites. Similarly our results, they demonstrated that α-ZOL was more estrogenic than ZEA and it almost as estrogenic as β-ZOL.

In conclusion the estrogenic effect of the three mycotoxins may be due because ZEA and its metabolites have a flexible structure (parveen et al. 2009), and they are able to bind to ER. And the higher RPE% of α-ZOL is due to a higher affinity for the ER. However, it is important to consider that estrogenic activity is not the only mechanism whereby ZEA, α-ZOL and β-ZOL produce toxicity

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DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

Se ha evaluado la citotoxicidad producida por la ZEA y sus metabolitos principales (α-ZOL y β-ZOL) de forma individual en las células hepáticas humanas HepG2 y en las células de ovario de hámster chino CHO-K1. Considerando que estas micotoxinas pueden encontrarse juntas en los alimentos y en el organismo y que puede producirse interacción entre ellas potenciándose el efecto tóxico, se evaluó la citotoxicidad de estas micotoxinas en combinaciones binarias y terciaria y se determinó el tipo de interacción que tenía lugar mediante el método de las isobolas.

Puesto que la ZEA se metaboliza a α-ZOL y β-ZOL se evaluó si se producía esta transformación en las células CHO-K1 y HepG2 así como la producción de otros derivados, tanto de la ZEA como del α-ZOL y β-ZOL.

Teniendo en cuenta el efecto tóxico de la ZEA y de sus metabolitos tanto de forma individual como combinada tras la exposición de las células CHO-K1 y HepG2 se estudiaron los posibles mecanismos por los que la ZEA y sus metabolitos pueden producir daño celular. Para ello, se determinó la producción de ROS. Tras observar que las micotoxinas producían un aumento de ROS, el siguiente paso fue plantearse si se originaba daño en el ADN en las células CHO-K1 y HepG2. Por otra parte, debido a que las células tienen su propio mecanismo de defensa antioxidante se determinó si la actividad de las enzimas antioxidantes CAT, SOD y de la GPX así como del sistema GSH no enzimático (antioxidante celular más importante), para determinar si se activaban los mecanismos de defensa celular.

Todos los efectos se estudiaron en dos tipos de células diferentes (CHO-K1 y HepG2).

Por último, como la ZEA y sus metabolitos ejercen su acción tóxica mediante la interacción con los RE produciendo una acción estrogénica, se

utilizaran las células MCF-7 que contienen dichos receptores con el fin de evaluar si éstas micotoxinas tenían suficiente actividad estrogénica como para alterar la proliferación normal de las mismas.

4.1. Citotoxicidad individual

Las células CHO-K1 y HepG2 se expusieron a diferentes concentraciones de las micotoxinas ZEA, α-ZOL y β-ZOL durante 24, 48 y 72h. La citotoxicidad se determinó mediante el método MTT que valora la alteración mitocondrial.

Los valores obtenidos se expresan como inhibición del crecimiento celular en el punto medio (IC_{50}). Después de 24h de exposición las valores de IC_{50} en las células CHO-K1 fueron >100, 33,00 ± 4,50 y >75μM para la ZEA, el α-ZOL y el β-ZOL, respectivamente. Tras 48h de exposición los valores son 60,30 ± 2,55, 32,00 ± 5,15, y 62,50 ± 4,56μM para la ZEA, el α-ZOL y el β-ZOL; tras 72h los valores de IC_{50} son 68,00 ± 2,55, 30,00 ± 5,50 y 55,00 ± 3,80μM para la ZEA, el α-ZOL y el β-ZOL, respectivamente. En cuanto a las células HepG2 se obtuvieron unos valores similares a las 24h de exposición, ya que los valores de IC_{50} fueron >100, 27,00 ± 4,00 y >100μM para la ZEA, el α-ZOL y el β-ZOL, respectivamente. Tras 48h de exposición los valores fueron 71,70 ± 5,70, 25,50 ± 4,20, y 45,00 ± 5,56μM para la ZEA, el α-ZOL y el β-ZOL, respectivamente y tras 72h los valores de IC_{50} fueron 70,00 ± 5,70, 20,60 ± 4,50 y de 38,40 ± 3,47μM para la ZEA, el α-ZOL y el β-ZOL, respectivamente.

Los resultados muestran que las tres micotoxinas ensayadas producen una acción tóxica alterando la función mitocondrial. La micotoxina que presenta una mayor toxicidad es el α-ZOL tanto en las células CHO-K1 como en las células HepG2. Según los valores de IC_{50} obtenidos en ambos tipos de células, la toxicidad sigue el siguiente orden α-ZOL > β-ZOL >

ZEA, siendo los metabolitos más tóxicos que la ZEA y siendo el α-ZOL el que produce una mayor toxicidad de los dos. La toxicidad aumenta con el tiempo de exposición siendo más tóxicas a 48 y 72h que a 24h También se observa como a las 48 y 72h tanto los metabolitos producen una mayor toxicidad en las células HepG2. Estos resultados son similares a los obtenidos por otros autores como Marin y col. 2010 que demostraron que la ZEA producía menos citotoxicidad que el α-ZOL y β-ZOL cuando expusieron neutrófilos a estas micotoxinas. Wang y col. 2014a también demostraron que el α-ZOL producía una mayor citotoxicidad que la ZEA cuando las células HepG2 se expusieron a estas micotoxinas. Ayed y col 2011 demostraron que el α-ZOL producía una mayor citotoxicidad que el β-ZOL en las células Hela, resultados similares a esto obtuvieron Salem y col. 2016 en las células HEK293 y Othmen y col. 2008 en las células Vero.

4.2. Citotoxicidad de micotoxinas en combinación

Debido a que las micotoxinas se pueden encontrar en combinación en los alimentos preocupa el peligro a la exposición de mezclas de micotoxinas. La presencia simultánea podría causar un aumento potencial en la toxicidad de las micotoxinas; esto puede suponer un mayor riesgo para el consumidor respecto a la exposición a micotoxinas de forma individual ya que se pueden producir efectos sinérgicos o aditivos (Wan y col 2013; Wang y col 2014a).

Por ello se evaluaron los efectos citotóxicos de la ZEA, α-ZOL y β-ZOL cuando se encuentran en combinaciones binarias y terciaria a las 24, 48 y 72h de exposición, en dos modelos celulares diferentes, células de ovario CHO-K1 y células hepáticas HepG2 mediante el método MTT.

Para determinar el tipo de interacción entre las diferentes micotoxinas de la mezcla se utilizó el método de las isobolas de Chou (2006) y Chou y

Talalay (1984). Este método ha sido ampliamente utilizado para ensayos sobre mezclas de medicamentos y se ha visto que también se puede usar para mezclas de micotoxinas. Este método permite determinar el tipo de interacción (efecto sinérgico, aditivo o antagonista) que tiene lugar entre las diferentes micotoxinas de una mezcla, sin embargo no nos informa sobre el mecanismo de acción por el cual se producen dichos efectos.

Este método tiene en cuenta tanto la citotoxicidad de las micotoxinas individuales y en combinación. Los valores de Índice de combinación (CI) obtenidos mediante este método indican si se produce efecto sinérgico (CI < 1), efecto aditivo (CI = 1) o efecto antagonista (CI > 1) (Geiger y col. 2016).

Los resultados obtenidos con este método mostraron que tanto las combinaciones binarias como la terciario en ambos tipos de células producen una mayor citotoxicidad que las micotoxinas cuando se encuentran de forma individual. De acuerdo con los valores de IC_{50}, la citotoxicidad de las combinaciones aumenta según el siguiente orden en las células CHO-K1 después de 24 h de exposición ZEA + α-ZOL < ZEA + β-ZOL = α-ZOL + β-ZOL = ZEA + α-ZOL + β-ZOL después de 48h y después de 72 h de exposición, ZEA + α-ZOL + β-ZOL < ZEA + α-ZOL = ZEA + β-ZOL < α-ZOL + β-ZOL. En cuanto a las células HepG2, después de 24, 48 y 72h de exposición ZEA + β-ZOL < ZEA + α-ZOL = ZEA + α-ZOL + β-ZOL < α-ZOL + β-ZOL. Hay que tener en cuenta que la mayor citotoxicidad producida en las combinaciones respecto a la exposición de las micotoxinas de forma individual se debe a que en las combinaciones hay una mayor concentración de micotoxinas. Respecto al tipo de interacción observado, la combinación de ZEA + β-ZOL muestra un efecto aditivo en todos los tiempos ensayados en ambos tipos de células tanto a concentraciones altas como a concentraciones bajas.

Mientras que la combinación ZEA + α-ZOL muestra mayoritariamente un efecto antagonista en las células HepG2 mientras que en las CHO-K1 muestra un efecto aditivo en todos los tiempos y concentraciones ensayados. Este mismo efecto se observa en la combinación binaria α-ZOL + β-ZOL en el caso de las células CHO-K1, pero en las células HepG2, se observa un efecto sinérgico. En cuanto a la combinación terciaria en el caso de las células HepG2 el efecto antagonista a concentraciones bajas se vuelve aditivo a concentraciones altas, mientras que en las células CHO-K1 se vuelve un efecto sinérgico. Resultados similares a estos fueron obtenidos poe Gao y col. 2016 cuando expusieron a las células Caco-2 a las combinaciones binarias ZEA + α-ZOL, ZEA + OTA α-ZOL + OTA obtuvieron resultados que iban desde un efecto antagonista hasta un efecto sinérgico de igual modo Wang y col. 2014a obtuvieron los mismo resultados cuando la combinación de ZEA + α-ZOL se expuso a las células HepG2. Los mismos resultados se produgeron en las células Caco-2 cuando Gao y col 2016 las expusieron a las mezclas terciarias ZEA + α-ZOL + AFM1 y ZEA + AFM1 + OTA. Cabe destacar que tanto los efectos sinérgicos como antagonistas en ambos tipos de células son muy débiles. Se puede suponer que los efectos sinérgicos observados se deben a la estructura lipófila de estas micotoxinas lo que les permite atravesar las membranas con gran facilidad aunque debido a que todas ellas presentan una estructura similar pueden competir con los mismos receptores produciendo efectos aditivos o antagonistas si se produce una saturación. Esto es debido a que los mecanismos son muy complejos.

4.3. Transformación de las micotoxinas

Teniendo en cuenta que se han observado una gran variedad de derivados de la ZEA y sus metabolitos, es interesante determinar los derivados producidos tanto en las células CHO-K1 como HepG2.

Se sabe que en la ZEA se puede reducir su grupo ceto del C7 y dar lugar a sus derivados reducidos, el α-ZOL y β-ZOL, que posteriormente se puede reducir el doble enlace dando lugar al α-ZAL y el β-ZAL. También se ha observado que se pueden formar derivados glucuronidados.

Además de los derivados anteriores, se han observado otros derivados de la ZEA y de sus metabolitos (Drzymala y col. 2015). Estos derivados de las micotoxinas pueden estar también presentes en los alimentos y en el organismo y pueden ser los causantes de la toxicidad de estas micotoxinas. Por todo ello, el siguiente objetivo fue saber si la ZEA se podía metabolizar a α-ZOL y β-ZOL en las células CHO-K1 y HepG2 tal y como se ha observado que ocurre *in vivo*.

Para estudiar los derivados producidos de la ZEA y sus metabolitos, las células CHO-K1 y las HepG2 se expusieron a 50 μM de ZEA, α-ZOL y β-ZOL durante 24 y 48h y posteriormente se analizó el medio de cultivo y las células por separado.

Para llevar a cabo este objetivo se realizó, una extracción liquida con acetato de etilo y se determinaron los metabolitos generados por LC/MS/LIT.

Se observa que la ZEA y sus metabolitos se degradan, esta degradación ocurre entre el 4,0 ± 1,1% al 81,3 ± 3,1% para la ZEA, del 8,6 ± 1,1% al 79,1 ± 2,2% para el α-ZOL y del 10,4 ± 2,2% al 85,0 ± 3,6% para β-ZOL en el caso de las células CHO-K1 y en las células HepG2 estos valores se encuentran entre el 77,6 ± 1,0% al 70,1% ± 0,8% para la ZEA, del 66,2 ± 1,1% al 64,7 ± 1,6% para el α-ZOL y del 46,8% ± 1,2 al 37,0 ±

0,9% para β-ZOL. Ni en las células CHO-K1 ni en las células HepG2 la ZEA se metaboliza a α-ZOL y β-ZOL pero se obtienen otros derivados. En las células CHO-K1 se obtiene productos de deshidrogenación tanto de la ZEA como de sus metabolitos. En el caso de la ZEA se identifica un derivado que ha perdido un grupo hidrógeno, en el α-ZOL y β-ZOL se obtiene un derivado que ha perdido dos grupos hidrógenos. En estas células, también se obtiene un producto de deshidroxilación del α-ZOL. En el caso de las células HepG2 también se obtienen derivados deshidroxilados, pero además se obtienen derivados hidroxilados, glucosidados y desmetilados para los metabolitos. Asimismo, en la ZEA se observan productos de desmetilación y de glucuronación. Para el α-ZOL y β-ZOL se observan derivados hidroxilados y derivados que han incorporado un grupo glucósido. Esto es importante ya que la citotoxicidad producida por estas micotoxinas puede ser debida en parte a estos metabolitos formados de la ZEA y sus derivados. Versilovskis y col. 2011 obtuvieron derivados diglucósidos y glucósidos de la ZEA, Pfeiffer y col. 2011 encontraron derivados Glucurónidos de la ZEA y Hildabrand y col. 2012 obtuvieron formas hidroxiladas de la ZEA. Además tanto Videmann y col 2008 como Pfeiffer y vol. 2011 encontraron derivados glucurónidos del α-ZOL y del β-ZOL.

4.4. Producción de especies reactivas de oxígeno (ROS): Estrés oxidativo

Teniendo en cuenta que la ZEA, el α-ZOL y el β-ZOL producen citotoxicidad el siguiente paso fue ver si esta citotoxicidad es debida a su capacidad para producir ROS (Sang y col 2016).

Para ello se utilizó el ensayo de la fluoresceína (para ver el aumento de ROS) y se midió el aumento de fluorescencia durante 2h a diferentes

intervalos. Los resultados de este estudio demuestran que la exposición de las células CHO-K1 y HepG2 a la ZEA, α-ZOL y β-ZOL de 0 a 120 minutos produce un aumento significativo de ROS respecto al control. Este aumento es mayor a tiempos bajos, produciendo un aumento de ROS a 0 min de 4,3, 5,3 y 4,6 veces el observado en el control en las células CHO-K1 en presencia de ZEA, α-ZOL y β-ZOL respectivamente y un aumento de 2, 7 y 2,5 veces respecto al control en las células HepG2 en presencia de ZEA, α-ZOL y β-ZOL respectivamente. Sin embargo a los tiempos más altos ensayados el aumento de ROS con respecto al control no es mayor de 1,7 veces en ambos tipos de células. Lo que demuestra que el mayor aumento de ROS se produce al principio y luego se estabiliza un poco.

El α-ZOL es el que produce un mayor aumento en las ROS en los dos tipos de células ensayados, y a medida que aumenta la concentración de las micotoxinas se observó un aumento de las ROS en ambos tipos de células. Similar a nuestros resultados, estudios previos han informado un aumento de la producción de ROS en diferentes células expuestas a ZEA a diferentes concentraciones (células HepG2, ZEA 100 μM, El Golli-Bennour y col., 2008, células CHO-K1, ZEA de 1-50 μM, Ferrer y col., 2009, células de granulosa porcina, ZEA de 15-60 μM, y col., 2015, células de hígado de L02, ZEA de 10-40 μM, Wu y col., 2014). Estos autores también encontraron que la producción de ROS es dependiente del tiempo (El Golli-Bennour et al., 2008, Ferrer et al., 2009). En este sentido, Ferrer et al (2009) informaron que la generación de ROS inducida por ZEA es más alta durante las primeras etapas de exposición (4 veces más que los controles), mientras que su después de 5 min (aumento de 2 veces). Otros autores mostraron también un aumento de ROS en diferentes concentraciones de α-ZOL y β-ZOL (Othmen y col. 2008 en células Vero;

Lu y col. 2013b en macrófagos; Salem y col. 2016 en las células HEK293; Salem 2017 en las células H9c2).

Estos resultados muestran la capacidad de la ZEA y sus metabolitos para producir un aumento de las ROS en ambos tipos de células. Por lo que se puede concluir que la producción de ROS es uno de los mecanismos por los que la ZEA y sus metabolitos producen citotoxicidad, confirmándose también el mayor potencial citotóxico del α-ZOL respecto a la ZEA y el β-ZOL.

4.5. Daño al ADN

Una vez observado que tanto la ZEA, el α-ZOL y β-ZOL son capaces de producir un aumento en las ROS tanto en las células CHO-K1 como en las células HepG2, el siguiente objetivo fue estudiar si este aumento de ROS puede producir un daño al ADN (Corcuera y col. 2015).

Para determinar si la ZEA y sus metabolitos producen daño en las cadenas de ADN se utilizó el ensayo del cometa a pH alcalino. Este ensayo detecta la rotura de las dobles cadenas y de las simples en los sitios alcalinos débiles y las roturas de las cadenas simples asociadas a reparaciones incompletas por escisión. Para ello las células se expusieron a 6,25, 12,5 y 25 μM de las micotoxinas durante 24h y se determinó el daño producido al ADN (Frankič y col. 2006). Los resultados obtenidos mostraron que tanto la ZEA como sus metabolitos producían daño en el ADN en ambos tipos de células. El α-ZOL es el que produce un mayor daño al ADN. Este daño fue del 43% y del 28% para el α-ZOL y β-ZOL en las células CHO-K1 a la concentración más alta ensayada respectivamente mientras que en las HepG2 este aumento fue del 9%, 45% y 30% para la ZEA, α-ZOL y β-ZOL, respectivamente. De este ensayo se puede concluir que estas micotoxinas producen daño al ADN, siendo el α-ZOL el que

produce un mayor daño en ambos tipos de células. El daño en ambas células ensayadas fue en orden creciente ZEA < β-ZOL < α-ZOL, por lo tanto los metabolitos de la ZEA producen una mayor genotoxicidad que su precursor. Además, este hecho coincide con el incremento de ROS tanto en las células CHO-K1 como en las HepG2 cuando se exponen a la ZEA, α-ZOL y β-ZOL. Estos resultados son consistentes con los obtenidos por otros autores usando diferentes tipos de células (células Vero, ZEA de 10-40 μM, Ayed-Boussema y col., 2007; células Caco-2, IC50 / 2, IC50 y 2 x IC50, ZEA , α-ZOL y β-ZOL, Abid-Essefi y col., 2009; Células HepG2, ZEA 100 μM, Gazzah y col., 2010; CCL13 células hepáticas, 25 - 200 μM ZEA, Kang y col., 2013; Células SH-SY5Y de neuroblastoma humano, ZEA de 25-100 μ M, Venkataramana y col., 2014)

4.6. Actividad estrogénica

Diversos estudios *in vivo* e *in vitro* han demostrado el potencial de actividad endocrina de determinados compuestos al interaccionar con los RE (Schirilò y col. 2011). Se conoce que tanto la ZEA como el α-ZOL y el β-ZOL pueden interaccionar con los RE debido a su estructura. Para determinar si estas micotoxinas tienen actividad estrogénica se utilizó las células MCF-7 porque contienen un elevado número de RE (Damaegdt 2016). El crecimiento dependiente de estrógenos en las células humanas MCF-7, convierte este ensayo en el método *in vitro* más sensible para pruebas estrogénicas (Drzymala y col. 2015). Este ensayo recibe el nombre de E-screen y es una técnica colorimétrica que determina el aumento en el número de células tras la exposición a la sustancia. Este método determina la densidad celular mediante la medición del contenido proteico celular.

Los resultados mostraron que a concentraciones bajas tanto la ZEA como el α-ZOL y el β-ZOL producen un aumento en la proliferación celular. La actividad estrogénica observada para el α-ZOL va desde 6,25 a 12,5μM y para la ZEA y β-ZOL va desde 6,25 a 25 μM. Se observó que el α-ZOL produce una mayor actividad a concentraciones más bajas. Esto contribuye a los resultados obtenidos en los anteriores ensayos en los que se mostraba que el α-ZOL es el que produce una mayor citotoxicidad.

Shier y col. (2001) clasificaron ZEA y 16 análogos estructurales de acuerdo con su potencia proliferativa en células MCF-7. Sus datos sugieren que el grupo funcional en la posición 6 tiene el mayor efecto sobre la estrogenicidad; α-ZOL (α-OH en la posición 6) demostró una mayor potencia que otras especies en estimular el crecimiento de células MCF-7. Según Parveen y col. (2009), este efecto puede deberse al hecho de que ZEA y sus metabolitos tienen una estructura flexible que es capaz de unirse a ER tan fuertemente como el estrógeno natural E2. La mayor potencia de α-ZOL en comparación con ZEA y β-ZOL refleja su mayor afinidad por ER en células MCF-7. Por otra parte, y en contraste con las hormonas, α-ZOL no se une a la proteína portadora aumentando así su capacidad de producir efectos tóxicos. Además, se observó que los valores de RPE% aumentaban a las concentraciones inferiores ensayadas, y no se observó alteración determinable o agonismo parcial de ZEA y sus metabolitos.

4.7. Sistemas de defensa celular

Una vez se ha observado que estas micotoxinas pueden producir citotoxicidad por aumento de las ROS, daño al ADN y actividad estrogénica, se estudia si tras la exposición a las micotoxinas, se activaban los mecanismos de defensa de las células frente al estrés oxidativo. Para

ello se determinaron los niveles de GSH ya que está considerado el mecanismo antioxidante celular más importante, al actuar como nucleófilo neutralizando una gran variedad de electrófilos en condiciones fisiológicas normales. La reducción del GSH tiene un papel fundamental para el mantenimiento de las adecuadas condiciones redox y su reducción es un indicador del estrés oxidativo (Deponte 2013; Mallebrera y col. 2014). Los resultados mostraron una disminución de los niveles de GSH tanto en las células CHO-K1 como en las HepG2. Esta disminución fue mayor para el α-ZOL en las células HepG2. El α-ZOL produjo una disminución del 36% en las células CHO-K1 y del 62% en las células HepG2 a la concentración más alta ensayada mientras que la ZEA y el β-ZOL produjeron una disminución del 26% y del 28% respectivamente en las células CHO-K1 y del 40% en las células HepG2. Estos resultados muestran que el GSH es un sistema de defensa celular tras la exposición de la ZEA y sus metabolitos. La disminución del GSH conlleva a una mayor susceptibilidad de las células al estrés oxidativo, lo que puede causar muchas alteraciones celulares. Esta mayor susceptibilidad es mayor en presencia del α-ZOL.

También se evaluó la actividad de las enzimas antioxidantes más activas frente a sustancias oxidantes, la superóxido dismutasa (SOD), la catalasa (CAT) y la glutatión peroxidasa (GPx). La actividad de estas enzimas se alteró por el α-ZOL especialmente en las células HepG2. Los resultados mostraron, un aumento en la actividad de la SOD y la GPx en ambas células, siendo el aumento de la GPx del 49%, 62% y 61% para la ZEA, α-ZOL y β-ZOL respectivamente, en las células CHO-K1 y del 50%, 95% y 90% respectivamente, en el caso de las HepG2. Mientras que la SOD aumentó un 48%, 69% y un 51% para la ZEA, α-ZOL y β-ZOL respectivamente, en las células CHO-K1 y un 50%, 70% y 55%, en el caso de las HepG2. Este aumento sugiere la activación de este sistema de

defensa celular contra la ZEA y sus metabolitos, lo que puede ayudar a disminuir la citotoxicidad producida por estas micotoxinas. Sin embargo por los resultados obtenidos anteriormente de citotoxicidad y de daño al ADN, estos sistemas no son suficientes para reparar el daño producido por esta micotoxinas

La CAT tiene una alta eficacia en la neutralización de el H_2O_2 por lo que constituye un sistema de defensa celular muy importante. Los resultados mostraron una disminución de la actividad de la CAT en ambas células tras la exposición a diferentes concentraciones de las tres micotoxinas. Esta disminución es mayor para los metabolitos que para la ZEA en ambas células. Esta disminución sugiere que las micotoxinas saturan la actividad de esta enzima.

Estudios anteriores han encontrado efectos similares en diferentes tipos de células expuestas a ZEA u otras micotoxinas de *Fusarium*. Sin embargo, hasta donde sabemos, no hay datos disponibles sobre los efectos oxidativos causados por la exposición a α-ZOL y β-ZOL. Lee y col. (2013) y Hassen y col. (2007) observaron que ZEA (200 μM) disminuyó los niveles de GSH en las células HepG2 (disminución del 71% y 33% en comparación con el control, respectivamente), mientras que Qin y col. (2015) observaron una disminución en la actividad de CAT cuando las células de la granulosa porcina fueron expuestas a ZEA (3-60 μM) durante 24 h. Resultados similares fueron obtenidos por otros autores, reportando niveles reducidos de GSH y / o aumento de GPx, SOD y CAT en diferentes células (CHO-K1, Caco-2, PK15, U937, HeK-293) expuestas a diferentes micotoxinas (beauvericina, BEA, fumonisina B1, FB1, desoxinivalenol, DON) (Mallebrera y col., 2014, Prosperini y col., 2013, Klaric y col., 2007, Costa y col., 2009, Dinu y col., 2011).

Los resultados muestran que el GSH, la GPx y la SOD son más eficaces neutralizando las ROS que la CAT en ambas células.

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CONCLUSIONES

5. CONCLUSIONES

1. La zearalenona y sus metabolitos producen citotoxicidad en las células de ovario de hámster chino (CHO-K1) y células de hepatoma humano (HepG2) dependiendo de la concentración y el tiempo de exposición. La mayor citotoxicidad se observa en las células HepG2 y el α-zearalenol es el que produce una mayor citotoxicidad en los dos tipos de células.
2. Las combinaciones binarias zearalenona + α-zearalenol, zearalenona + β-zearalenol y α-zearalenol + β-zearalenol presentan principalmente efecto aditivo a los tres tiempos de exposición ensayados en los dos tipos de células. Mientras que la combinación terciaria presenta sinergismo a las 24, 48 y 72h de exposición.
3. Las células CHO-K1 y HepG2 no metabolizan la zearalenona en α-zearalenol y β-zearalenol, pero se detectan otros metabolitos diferentes
4. El estrés oxidativo es uno de los mecanismos implicados en la toxicidad producida por zearalenona y sus metabolitos en las células CKO-K1 y HepG2. La zearalenona y sus metabolitos producen un incremento de especies reactivas de oxígeno, dependiendo de la concentración y tiempo. La zearalenona y β-zearalenol producen un mayor aumento de especies reactivas de oxígeno en las células CHO-K1 y el α-zearalenol en las células HepG2.
5. La zearalenona y sus metabolitos dañan el ADN. El daño aumenta dependiendo de la concentración. El α-zearalenol produce un mayor daño al ADN que la zearalenona y β-zearalenol. Este daño es mayor en las células HepG2.

6. En las células de adenocarcinoma de mama humano MCF-7 se observa un aumento en la actividad estrogénica a concentraciones bajas, siendo el α-zearalenol el que produce un mayor efecto. A concentraciones altas se observa citotoxicidad.
7. Al aumentar la concentración de zearalenona, α-zearalenol y β-zearalenol aumenta la actividad enzimática de la superóxido dismutasa, glutatión peroxidasa y disminuyen la actividad de la catalasa y glutatión, demostrando la activación de los mecanismos de defensa frente a la citotoxicidad de dichas micotoxinas. Este efecto es mayor en las células HepG2. El α-zearalenol produce una mayor actividad que la zearalenona y el β-zearalenol.
8. La zearalenona y sus metabolitos son un problema de salud pública por la complejidad de sus efectos tóxicos. Por lo que un mayor conocimiento de sus mecanismos de acción contribuiría a mejorar la salud de los consumidores.

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ANEXO

7. ANEXO: Metodología

1. Reactivos y patrones

Todos los reactivos y componentes de los cultivos celulares utilizados: medio de cultivo (HAM-F12), medio de cultivo (DMEM), penicilina, estreptomicina, fungizona, tampón fosfato (HEPES), bromuro de 3[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio (MTT), 17β-estradiol (E2), aminoácidos no esenciales (NEAA) tampón fosfato salino (PBS), glucosa, dimetil sulfóxido (DMSO), tritón X-100, 2',7'-diclorodihidrofluoresceína diacetato (H_2-DCFDA), o-ftataldialdehído (OPT), yoduro de propidio (PI), ácido etilendiaminotetraacético sódico (Na_2-EDTA), agarosa, agarosa de bajo punto de fusión (LMA), sulforodamina B (SRB), ácido tricloroacético (TCA), zearalenona (ZEA) (pureza: ≥ 97%; peso molecular 318,36 g/mol), α-zearalenol (α-ZOL) (pureza: ≥ 97%; peso molecular: 320,38 g/mol) y β-zearalenol (β-ZOL) (pureza: ≥ 97%; peso molecular: 320,38 g/mol) se obtuvieron de Sigma-Aldrich (Madrid). El bicarbonato sódico ($NaHCO_3$) se obtuvo de Guinama (Valencia) y el suero fetal bovino (SFB) de Cambrex Company (Bélgica). El agua desionizada (resistividad <18 µOms/cm) se obtuvo usando un sistema de purificación Milli-Q (Millipore, Bedford, MA, EEUU).

Las soluciones de micotoxinas se prepararon en metanol y se mantuvieron a -20°C en oscuridad.

Las concentraciones finales ensayadas se obtuvieron añadiendo medio de cultivo a las soluciones de micotoxinas siendo la concentración final de metanol del 1% (v/v). El contenido de proteína total (µg/ml) se determinó por el método Bradford (Bio-Rad DC Protein Assay nº catálogo 500-0116 http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf).

Para el ensayo analítico, la cromatografía líquida acoplada espectrometría de masas (LC-MS) (Applied Biosystems/MDS SCIEX Q

TRAP TM espectrómetro de trampa de iones linear, Concord, Ontario, Canadá) con una columna Geminis (150 x 4,6 mm, 5 µm Phenomenex Torrance, CA).

2. Cultivos celulares

Las células CHO-K1 se siembran en medio HAM-F12 suplementado con 25 mM de tampón HEPES (pH 7,4), 10% de SFB inactivado, 100 U/mL de penicilina, 100 g/mL de estreptomicina y 0,1% (v/v) de fungizona. Se cultivan en condiciones asépticas en monocapa, en discos de cultivo de poliestireno de 9 cm^2, en una atmósfera de 37 ºC con un 5% de CO_2 y un 95% de humedad relativa.

Las células HepG2 y las células MCF7 se cultivaron en frascos a pH 7,4, 37 ºC, 5% CO_2 and 95% de humedad. Las células MCF7 se mantuvieron en medio DMEM con 15 mg/L de rojo fenol y suplementado con 5% FBS, 1% glutamina, 1% NEAA, 1% piruvato y 0,1% insulina. Las células HepG2 crecieron usando el medio DMEM suplementado con 25 mM HEPES, 10% FBS, 100 U/ml penicilina y 100 mg/ml estreptomicina. La ausencia de micoplasma se determina usando el Micoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

3. Ensayo de viabilidad celular

El ensayo de proliferación celular del MTT se realiza siguiendo el procedimiento de Ruiz y col., (2006). Las células CHO-K1 y HepG2 se cultivan en placas de 96 pocillos añadiendo 200 µL/pocillo de una suspensión que tiene una densidad de $2x10^4$ células/pocillo. Después de que las células alcanzan una confluencia del 65%; determinada por microscopio, el medio de cultivo se reemplaza por medio fresco que contiene una serie de diluciones de cada micotoxina y se dejan incubar

durante 24, 48 y 72 h. Estas diluciones son para ZEA de 12,5, 18,7, 25, 37,5, 50, 75 y 100 µM; para el α-ZOL de 6,25, 9,36, 12,5, 18,7, 25, 37,5, 50, 75 y 100 µM y para β-ZOL de: 6,25, 9,36, 12,5, 18,7, 25, 37,5, 50 y 75 µM. Tras una incubación de las células con las micotoxinas durante 24, 48 y 72 h, el medio de cultivo con las micotoxinas se elimina y reemplaza por medio fresco que contiene 50 µL (5 mg MTT/ml PBS estéril) de solución MTT. Después de 4 h de incubación en oscuridad y a 37 ºC, se lavan los pocillos con PBS y se fija el colorante durante 10 min con DMSO seguido de un tampón de glicina Sorensen. La absorbancia se mide a 570 nm en un lector automático de placas multipocillo (MultisKanEX, Labsystem, Helsinki, Finlandia).

4. Diseño experimental y evaluación de los efectos de las combinaciones de micotoxinas

Las soluciones de ZEA, α-ZOL y β-ZOL preparadas y descritas previamente se usaron en combinaciones de dos micotoxinas (ZEA + α-ZOL; ZEA + β-ZOL y α-ZOL + β-ZOL) y de las tres micotoxinas juntas (ZEA + α-ZOL + β-ZOL). Las concentraciones utilizadas en las mezclas de 2 y 3 micotoxinas fueron, para la ZEA: de12,5 µM a 50 µM; α-ZOL y β-ZOL: de 6,25 µM a 25µM.

Las células CHO-K1 y HepG2 se exponen a diluciones seriadas de cada micotoxina individualmente y con una relación constante, en sus combinaciones binarias y ternarias de (ZEA+α-ZOL proporción: 2:1; ZEA + β-ZOL proporción: 2:1; α-ZOL+β-ZOL proporción: 1:1), siendo las concentraciones inferiores a sus valores de IC_{50} individuales. Cinco diluciones de estas micotoxinas en las proporciones descritas anteriormente, más un control se ensayan en tres experimentos

independientes. La viavilidad celular de las combinaciones binarias y terciarias de micotoxinas se ensaya por el método MTT.

Para determinar el tipo de interacción producido por la combinación de ZEA y sus metabolitos, se utiliza el método de las isobolas, que utiliza la ecuación de efecto-medio/índice de combinación (CI) descrito por Chou (2006) y Chou y Talalay (1984). Este método, se basa en el principio de la ley de acción de masas que demuestra que hay una relación unívoca entre la dosis de una mezcla de sustancias y el efecto que produce, independientemente del número de sustancias en la mezcla y del mecanismo de acción celular. Con este método se calculan las curvas dosis-efecto para cada compuesto y sus combinaciones en múltiples concentraciones diluidas usando la ecuación de efecto medio:

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

Donde D es la concentración de la micotoxina, D_m es la dosis media efectiva (ej., IC_{50}, EC_{50}, o LD_{50}) que inhibe el 50% de la viabilidad celular, *fa* es la fracción afectada por la concentración D (ej., porcentaje de inhibición/100), *fu* es la fracción no afectada (por lo tanto, *fa* = 1 - *fu*), y m es el coeficiente que indica el tipo de relación dosis-efecto que tiene lugar. Así cuando m = 1, m > 1, y m < 1 indica que la curva dosis-efecto es hiperbólica, sigmoidal o negativo sigmoidal respectivamente (Chou y Talalay, 1984). Por lo tanto, el método tiene en cuenta los parámetros de potencia (Dm) y de la pendiente de la curva (m).

La ecuación de efecto medio para compuestos individuales se puede modificar y aplicar a múltiples micotoxinas. Así por ejemplo, en el caso de una mezcla binaria la ecuación se convierte en:

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

Cuando dos micotoxinas se combinan y someten a varias diluciones, la mezcla de las dos micotoxinas se comporta como una tercera micotoxina

en la relación dosis efecto. Por lo tanto, y= log $[(fa)_{1,2}/(fu)_{1,2}]$ frente a x = log $[(D)_1+(D)_2]$ donde se obtiene los valores de $m_{1,2}$, $(D_m)_{1,2}$, y $r_{1,2}$.

Chou y Talalay (1984) introdujeron el término de índice de combinación (CI) para cuantificar el tipo de interacción de dos compuestos:

$CI = D_1/(D_x)_1 + D_2/(D_x)_2 = (D)_1/(D_m)_1[fa/(1-fa)]^{1/m1} + (D)_2/(D_m)_2[fa/(1-fa)]^{1/m2}$

Donde en el denominador $(Dx)_1$ es para el compuesto 1 "individual" que inhibe un x% del crecimiento celular, y $(Dx)_2$ es para el compuesto 2 "individual" que inhibe un x% del crecimiento celular.

Y la ecuación general para la combinación de n-compuestos y una inhibición de *x*% de los mismos se convierte en:

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

Donde (CI) es el índice de combinación para n compuestos (ej., micotoxinas) a un *x*% de inhibición (ej., inhibición de la proliferación); $(D_x)_{1-n}$ es la suma de la concentración de los n compuestos que ejerce *x*% de inhibición de la mezcla, $\{[D_j]/\sum^{n}_{1}[D]\}$es la proporción de la concentración de cada uno de los n compuestos que ejerce el *x*% de inhibición en la combinación; y $(D_m)_j \{(fax)_j/[1-(fax)_j]\}^{1/mj}$ es la concentración de cada compuesto individual que ejerce un *x*% de inhibición. En esta ecuación, CI <1, =1 y >1 indica sinergismo, efecto aditivo y antagonismo, respectivamente.

5. Determinación de la ZEA, α-ZOL y β-ZOL y sus derivados por LC-MS-LIT

Para determinar la ZEA y sus metabolitos en las células CHO-K1 y HepG2 se utilizó la cromatografía líquida acoplada a espectrometría de masas con trampa de iones lineal (LC/MS/LIT). Un total de 10000 células por cm^2 se cultivaron en placas de cultivo de poliéster de 9 cm^2 con medio de cultivo hasta obtener la confluencia deseada. Se adicionó 50 μM de ZEA, α-ZOL and β-ZOL y se incubó durante 24 y 48 h. Después, el medio y las células se recogieron, extrajeron y analizaron por LC/MS/LIT. Las células se recogieron en un eppendorf, se añadió un 1 μL de medio de cultivo y se puso en ultrasonidos durante 3h para romperlas. Después, se realiza la extracción con etilacetato y el extracto se seco en turbobap y se redisuelta en 1mL de metanol. Respecto al medio de cultivo se realiza el mismo proceso de extracción.

Las condiciones de la LC se establecieron usando un flujo constante a 0,3 mL/min, con ácido fórmico (HCOOH)-agua al 0,1% (fase móvil A) y acetronitrilo-HCOOH al 0,1% (fase móvil B). El gradiente empleado fue: 5 min con un 5% de la fase B, 15-25 min con un 90% B, 35-40 min con un 5% B. El análisis de la ZEA, α-ZOL and β-ZOL se llevo a cabo usando la técnica de LC-MS-LIT con el siguiente proceso: mediante la comparación de los tiempos de retención y los espectros UV de las muestras purificadas y los estándares puros usando un rango *m/z* de 300 a 350 Da para obtener el espectro general de degradación; la utilización de espectrómetro de masas asociado a la detección con la LIT, permite obtener una buena caracterización de los compuestos aislados (Juan y col. 2012). El método, para la detección y cuantificación de las micotoxinas que son objetivo de este estudio se valido con un método cuantificativo de acuerdo con la EU Commission Decision, 2002/657/EC (Comision decisión 2002) y los

parámetros que se tuvieron en cuenta para este propósito fueron: linealidad, exactitud, precisión (repetibilidad y reproducibilidad) y sensibilidad. La linealidad se evaluó usando unas curvas de calibración estándar que se prepararon para cada micotoxina usando los patrones de cada micotoxina. La precisión se evaluó mediante el cálculo de las recuperaciones de compuestos individuales. La precisión intra-día se evaluó mediante el cálculo de la desviación estándar relativa (RSDr), calculada a partir de los resultados obtenidos de las seis repeticiones por concentración en un solo día. La precisión inter-día se calculó mediante la RSDr calculada a partir de los resultados obtenidos en condiciones de reproducibilidad por los resultados obtenidos de cada concentración durante 6 días. La sensibilidad se evaluó por el límite de detección (LD) y límite de cuantificación (LQ).

6. Producción de ROS

La producción de ROS intracelular se monitorizó en células CHO-K1 y HepG2 mediante la adición de H_2-DCFDA. Este método es excepcionalmente sensible y proporciona una medida directa del estrés oxidativo general, con la detección de oxidantes intracelulares. La H_2-DCFDA es absorbida por las células y desacetilada por esterasas intracelulares, quedando atrapada en el interior de la célula. El método se llevo a cabo según Ruiz-Leal y George (2004). Brevemente, 2×10^4 células/pocillo se sembraron en placas negras de 96 pocillos con el medio de cultivo. Una vez las células alcanzaron la confluencia necesaria, el medio de cultivo se reemplazó por medio nuevo al que se adicionó 20 μM de DCFH-DA durante 20 min. El medio con DCFH-DA fue retirado y las células se lavaron con PBS esto antes de la adición del medio/1 % MeOH (control), medio con la ZEA o medio con sus metabolitos. El aumento de la

fluorescencia se midió a intervalos de hasta 2 h a las longitudes de onda de excitación y emisión de 485 y 535 nm, respectivamente. Se hicieron veinticuatro repeticiones. Los resultados se expresan como el aumento de la fluorescencia con respecto al control. Las determinaciones se realizaron en tres experimentos independientes

7. Ensayo del cometa alcalino

Las células CHO-K1 y HepG2 se sembraron en placas de cultivo de 6 pocillos a una densidad de 3,4 x 10^{5} células por pocillo y se esperó 24h hasta alcanzar la confluencia. Se eliminó el medio de cultivo y se añadió la ZEA y sus metabolitos (α-ZOL y β-ZOL) a las concentraciones de 6,25, 12,5 y 25 μM en medio nuevo y se mantuvo durante 24 h. El ensayo del cometa alcalino se realizó para determinar las roturas de la cadena de ADN de acuerdo con el método descrito por Mallebrera y col. (2016). Los resultados se expresan como el porcentaje de ADN en la cola (%), calculado de acuerdo con la ecuación: % DNA en la cola = (intensidad total de la cola / intensidad total del cometa) x 100. Intensidad total del cometa = longitud de la cabeza + longitud de la cola. Las determinaciones se realizaron en tres experimentos independientes.

8. E-screen

El ensayo del E-screen se llevó a cabo de acuerdo con el método descrito por Körner y col. (1999) y modificado por Schirilò et al. (2009). En resumen, las células MCF-7 se tripsinizaron y se sembraron en placas de 96 pocillos con medio de ensayo a una concentración de 2 x 10^{3} células / pocillo. Las células se dejaron unir durante 24 h, y el medio de siembra se retiró, se lavó con PBS y se reemplazó con medio de ensayo que contenía diluciones en serie de ZEA (de 12,5 a 100 μM), α-ZOL y β-ZOL (de 6,5 a

100 µM), E2 como control positivo (10 - 8 - 10 - 3 M) y control negativo (disolvente). El bioensayo finalizó al día 6. Se ha demostrado que E2 induce proliferación máxima a una concentración de 10-9 M.

Estudios previos en nuestro laboratorio indican que la fase exponencial tardía de la proliferación de células MCF-7 en respuesta a la concentración de E2 se alcanza después de 144 h (6 días). Después del tiempo de incubación, el medio se aspiró cuidadosamente para evitar el desprendimiento celular y las células se incubaron a 4ºC durante 1 h en TCA al 10% (p / v). El TCA se retiró y los pocillos se lavaron bajo una corriente suave de agua del grifo y se secaron completamente al aire. Las células fijadas con TCA se tiñeron con SRB al 0,4% (p / v) disuelto en ácido acético al 1%. Después de la incubación, el sobrenadante se descartó y el colorante no unido se eliminó enjuagando los pocillos con ácido acético al 1%. Después de un secado a fondo, el colorante unido se solubilizó con 10 mM de base Tris, pH 10,5, durante 20-30 min. La densidad óptica (DO) se midió a 570 nm con un filtro de referencia a 690 nm usando un fluorímetro (lector de placas Multiskan MCC / 340, Thermo Fisher Scientific, Marietta, OH, EE.UU.). Se llevaron a cabo tres experimentos independientes.

Lo que se evalua en el ensayo del ensayo E-screen es la proliferación celular en relación con el control sin hormonas. El efecto proliferativo (PE) de una muestra es la relación entre el valor máximo de OD que se obtuvo con cada micotoxina o E2 y el número de células del control negativo: PE = número máximo de células número / número de células control negativo.

La actividad estrogénica de las muestras se evaluó determinando el efecto proliferativo relativo (RPE%), que compara la máxima proliferación inducida por la micotoxina con la inducida por E2:

$$RPE\% = [(PE\text{-}1)_{mycotoxin}/(PE\text{-}1)_{E2}] \times 100$$

9. Determinación del GSH

Para determinar los niveles de GSH en las células CHO-K1y HepG2 expuestas a ZEA y sus metabolitos, 3 x 10^5 células/pocillo se sembraron en placas de 6-pocillos. Después de que las células alcanzan la confluencia, se eliminó el medio de cultivo y las células se expusieron a la ZEA y sus metabolitos (6.25, 12.5 and 25 μM) durante 24 h. Tras dicho tiempo, el medio se retiró y las células se lavaron dos veces con PBS y se recogieron en 0,5 mL de 20 mM Tris y 0,1% Tritón.

Para la determinación del GSH reducido se utiliza el método de Maran y col. (2009) adaptado. Brevemente, se sembró 10 μL de las células homogenizadas anteriormente en placas negras de 96-pocillos con 200 μL de tampón GSH (0.1 M fosfato 5 mM EDTA) a pH 8 y 10 ML de solución de OPT (1 mg/ml disuelta en metanol), mezcladas e incubadas en oscuridad a temperatura ambiente durante 15 min. La concentración de GSH se determina utilizando un lector de microplacas (Wallace Victor2, 1420 Multilabel Counter, Perkin Elmer, Turku, Finland) con una longitud de onda de excitación y de emisión de 345 y 425 nm, respectivamente. Los niveles de GSH se expresaron como μg GSH/mg de proteína. Las determinaciones se realizaron por triplicado.

10. Determinación de la actividad enzimática

Para determinar los niveles de la GPx, SOD y CAT en las células CHO-K1y HepG2 expuestas a ZEA y sus metabolitos, se siembran 3 x 10^5 células/pocillo en placas de 6-pocillos. Después de que las células alcanzan la confluencia, se eliminó el medio de cultivo y las células se expusieron a la ZEA y sus metabolitos (6.25, 12.5 and 25 μM) durante 24

h. Tras dicho tiempo, el medio se retiró y las células se lavaron dos veces con PBS y se recogieron en 0,5 mL de 20 mM Tris y 0,1% Tritón.

La actividad de la GPx se determinó de forma espectrofotométrica usando H_2O_2 como sustrato de la GPx dependiente de selenio, seguido de la oxidación del NADPH a 340 nm durante los primeros 2 min en un par de reacciones enzimáticas acoplada con la GR, descritas por Maran y col. (2009). En un de volumen final de 1 mL, se adicionaron 500 µL de 0.1 M tampón fosfato (pH 7.5, 1 mM EDTA, 2 mM NaN_3 y 0.1% Triton X-100), 250 µL de agua ultrapura, 100 µL de 20 mM de GSH, 20 µL de 0.2 mM NADPH, 2,5 U de GR recién preparada, y 50 µL de 5 mM de H_2O_2. 50 µL de la muestra de células homogenizadas se adicionó a la mezcla. Una unidad de GPx puede reducir 1 µmol de GSSG por min a pH 7.5. Los ensayos se realizaron a 25 °C durante 2 min en termocirculación (Perkin Elmer UV-VIS espectrómetro Lambda 2 versión 5.1). La actividad enzimática de la GPx se calculó usando la absorción molar de NADPH (6.22 mM^{-1} cm^{-1}), y se expresó como nmol de actividad de GPx/min/mg de proteína. Las determinaciones fueron realizadas por triplicado.

La actividad de la SOD se determinó utilizando el kit Ransod (Randox Laboratories, United Kingdom) adaptado a cubetas de 1.5 ml. La actividad de la SOD se monitorizó a 505 nm durante 3 min a 37 ºC en termocirculación (Perkin Elmer UV-VIS espectrómetro Lambda 2 versión 5.1). La actividad enzimática de la SOD se expresó como unidades de SOD por mg de proteína. Las determinaciones fueron realizadas por triplicado.

La actividad de la CAT se determinó según Espín y col. (2014), con pequeñas modificaciones. Brevemente, 50 µL de la suspensión de células homogenizadas se mezcló con 950 µL de 0.05 M de NaH_2PO_4 y 500 µL de 0.03 M de H_2O_2. La cinética de la descomposición enzimática del H_2O_2 se

determinó como la disminución de la absorbancia a 240 nm durante 2 min en el espectrofotómetro (super aquarious CECIL CE 9500). La actividad enzimática de la CAT se calculó usando la absorción molar del H_2O_2 (43.6 mM^{-1} cm^{-1}), y expresada como µmol de H_2O_2/min/mg de proteína. Las determinaciones fueron realizadas por triplicado.

11. Determinación del contenido de proteína total

El contenido de proteína celular total se ensayo usando el kit Bio-Rad DC Protein Assay; catalog number 500-0116 (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf). La concentración de proteína se midió a 690 nm.

12. Análisis estadístico

El análisis estadístico se llevó a cabo usando el paquete de software SPSS versión 19.0 (SPSS, Chicago, IL, USA). Los resultados se expresaron como media ± (desviación estándar) SD. Se realizó la t-Student's para muestras pareadas y las diferencias entre los grupos se analizaron estadísticamente con ANOVA seguido del test de Tukey HSD para comparación múltiple. El valor de $P \leq 0.05$ se consideró estadísticamente significativo.