VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia i Ciències de l'Alimentació

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

Programa de Doctorado con Mención hacia la Excelencia en Ciencias de la Alimentación

TOXIC EFFECTS OF MYCOTOXINS, ACRYLAMIDE AND ITS COMBINATIONS ON HUMAN NEUROBLASTOMA SH-SY5Y CELLS.

Efectos tóxicos de micotoxinas, acrilamida y sus combinaciones en células de neuroblastoma humano SH-SY5Y.

Tesis Doctoral Internacional

Presentada por:

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Dirigida por:

Dra. Ana Juan García

Dra. Houda Berrada Ramdani

Valencia, Mayo 2025

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia i Ciències de l'Alimentació

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CERTIFICAN QUE:

La Graduada en Nutrición Humana y Dietética **Doña Luna Bridgeman Torres** ha realizado bajo su dirección el trabajo **"Efectos tóxicos de micotoxinas, acrilamida y sus combinaciones en células de neuroblastoma humano SH-SY5Y"**, y autorizan su presentación para optar al título de Doctora con mención internacional por la Universidad de Valencia.

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

Valencia, Mayo, 2025.

Dra. Ana Juan García

Dra. Houda Berrada Ramdani

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To whom it may concern

I, undersigned Isabelle Severin, associate professor in food toxicology at Institut Agro Dijon, certify that Ms. Luna Bridgeman Torres has successfully completed a four-month internship at our laboratory from April 1, 2024, to July 31, 2024. During her time with us, Ms. Bridgeman Torres demonstrated excellent integration into our team and quickly mastered the protocol for the gene mutation test on bacteria, performing it independently.

Ms. Bridgeman Torres was able to carry out the appropriate experiments for the continuation of her thesis work with a high degree of competence and precision. She is a pleasant individual who maintained a balance of independence while remaining receptive to guidance from her supervisor. Her scientific rigor and dedication were evident throughout her stay.

We were very pleased with Ms. Bridgeman performance and have no hesitation in recommending her for any future academic or professional opportunities.

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I.SEVERIN

“El camino al paraíso comienza en el Infierno” Dante Alighieri

Pues nada, aquí estamos. Llegamos. Finalmente.

Ha sido largo, intensamente largo, pero bueno, ya sabéis eso de que lo importante es el camino, no la meta... pura basura. Podemos decir, al menos por esta vez, que después de todo esto soy doctora. *¡Yuju!*

Y, sinceramente, todo lo que quiero ahora es convertirme en *tradwife* y no volver a trabajar jamás. ¿Valió la pena? No. ¿Lo volvería a hacer? Definitivamente sí.

¿Y sabéis por qué? Porque por mucho que me pese admitirlo, ese caos de decisiones cuestionables, cafés/tés recalentados y estrés celular dio lugar a algo que sí merece la pena: la gente.

Esa gente que vino para quedarse. Que, de alguna manera, apareció en medio del desorden y terminó acompañándome a lo largo de estos años. Así que sí, si volviera atrás, no cambiaría nada. Ni un detalle. Porque no quiero perder ni una sola de estas amistades.

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Y por supuesto, a mi familia. Por escucharme, orientarme y apoyarme cuando más lo necesitaba.

Por estar ahí, incluso cuando ni yo sabía dónde estaba. Os quiero.

— Lu

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List of abbreviations

AA: Acrylamide,

DNA: Deoxyribonucleic acid,

AMPK: AMP-activated protein kinase,

ATP: adenosine triphosphate,

BAX: Bcl-2-associated Protein X,

BCL2: B-cell leukemia/lymphoma 2 protein 2,

BDNF: brain-derived neurotrophic factor,

BEA: Beauvericin,

CASP3: Aspartic acid cysteine-protease,

CCK8: Cell Counting Kit-8,

CHOP: homologous protein C/EBP,

COX: prostaglandin-endoperoxide synthase,

CREB: binding to the response element of the cAMP,

DCFH-DA: dichloro-dihydro-fluorescein diacetate,

DON: Deoxynivalenol,

eIF2α: Eukaryotic initiation factor 2 alpha,

ENN's: enniatin,

Erβ: Estrogen receptor beta,

FAK: focal adhesion kinase,

FB1: Fumonisin B1,

CF: Flow cytometry,

GRP78: glucose-regulated protein 78,

GSH: glutathione,

GSK-3β: glycogen synthase kinase-3β,

HSPs: Heat Shock Protein,

IC_{20}: concentration causing a 20% reduction in protein,

IC_{50}: 50% inhibition concentration,

iNOS: inducible nitric oxide synthase,

JAK-STAT: janus kinase- signal transducer and transcription activator,

JNK: c-Jun N-terminal kinase,

KEAP1: Kelch-like ECH-associated protein 1,

LDH: lactate dehydrogenase,

LPO: lipid peroxidation,

MAPKS: mitogen-activated protein kinases,

MDA: malondialdehyde,

MMP: Mitochondrial membrane potential,

MTT: (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide),

ND20: concentration that causes neurite degeneration of 20%.

NF-κB: nuclear factor κB,

NI20: 20% reduction in network formation.

NRF1: Nuclear respiratory factor-1,

NRF2: nuclear factor erythroid 2–factor 2 related to factor 2,

OTA: Ochratoxin A,

Q: Phospho,

P53: p53 tumor protein,

PERK: protein kinase,

PERK/eIF2α: RNA-like endoplasmic reticulum protein kinase - Eukaryotic initiation factor 2 alpha,

PGC-1α: peroxisome proliferator-activated receptor-gamma coactivator,

PI3K: phosphatidylinositol 3-kinase,

Pyk2: RNA-type proline-rich tyrosine kinase 2 (PKR)/pancreatic RE kinase**,**

qPCR: quantitative polymerase chain reaction,

ROS: reactive oxygen species.

SH-SY5Y: neuroblastoma cell line.

Sirt 1: NAD-dependent sirtuin-1 deacetylase

SNAP: Synaptic vesicle protein S-nitroso-N-acetyl penicillamine

TFAM: mitochondrial transcription factor A,

U-1240 MG: Human glioblastoma astrocytoma cells.

Z- DEVD-fmk: Z-D(OMe)E(Ome)VD(OMe)-FMK. Z-DEVD-fluoromethyl ketone. Z-DEVD-fluoromethyl ketone. specific caspase-3 inhibitor,

ZEA: Zearalenone

α-ZEL: alpha-zearalenol

β-ZEL: beta-zearalenol.4.

Lista de abreviaturas

AA: Acrilamida,

ADN: Ácido desoxirribonucleico,

AMPK: proteína quinasa activada por AMP,

ATP: trifosfato de adenosina,

BAX: Proteína X asociada a Bcl-2,

BCL2: proteína 2 de leucemia/linfoma 2 de células B,

BDNF: factor neurotrófico derivado del cerebro,

BEA: Beauvericina,

CASP3: cisteína-proteasa de ácido aspártico,

CCK8: kit de recuento de células-8,

CHOP: proteína homóloga C/EBP,

COX: prostaglandina-endoperóxido sintasa,

CREB: unión al elemento de respuesta del AMPc,

DCFH-DA: diacetato de dicloro-dihidro-fluoresceína,

DON: Deoxinivalenol,

eIF2*α*: Factor de iniciación eucariota 2 alfa,

ENN's: enniatina,

Erβ: Receptor de estrógeno beta,

FAK: quinasa de adhesión focal,

FB1: Fumonisina B1,

FC: Citometría de flujo,

GRP78: proteína 78 regulada por glucosa,

GSH: glutatión,

GSK-3β: glucógeno sintasa quinasa-3β,

HSPs: proteína de choque térmico,

IC_{20}: concentración que causa una reducción de proteínas del 20%,

IC_{50}: concentración de inhibición del 50%,

iNOS: óxido nítrico sintasa inducible,

JAK-STAT: quinasa janus- transductor de señal y activador de la transcripción,

JNK: c-Jun N-terminal quinasa,

KEAP1: Proteína 1 asociada a ECH similar a Kelch,

LDH: lactato deshidrogenasa,

LPO: peroxidación lipídica,

MAPKS: proteínas quinasas activadas por mitógenos,

MDA: malondialdehído,

MMP: Potencial de membrana mitocondrial,

MTT: (bromuro de 3-[4,5-dimetiltiazol-2-il]-2,5 difenil tetrazolio),

ND_{20}: concentración que causa una degeneración de neuritas del 20%.

NF-κB: factor nuclear κB,

NI_{20}: reducción del 20% en la formación de redes.

NRF1: Factor respiratorio nuclear-1,

NRF2: factor nuclear eritroide 2–factor 2 relacionado con el factor 2,

OTA: Ocratoxina A,

P: Phospho,

P53: proteína tumoral p53,

PERK: proteína quinasa,

PERK/eIF2α: proteína quinasa retículo endoplásmico similar al ARN - Factor de iniciación eucariota 2 alfa,

PGC-1α: coactivador del receptor-gamma activado por el proliferador de peroxisomas,

PI3K: fosfatidilinositol 3-quinasa,

Pyk2: tirosina quinasa 2 rica en prolina tipo ARN (PKR)/quinasa RE pancreática**,**

qPCR: reacción en cadena de la polimerasa cuantitativa,

ROS: especies reactivas de oxígeno.

SH-SY5Y: línea celular de neuroblastoma.

Sirt 1: Sirtuina-1 desacetilasa dependiente de NAD

SNAP: proteína de vesícula sináptica S-nitroso-N-acetil penicilamina

TFAM: factor de transcripción mitocondrial A,

U-1240 MG: Glioblastoma humano células de astrocitoma.

Z- DEVD-fmk: Z-D(OMe)E(Ome)VD(OMe)-FMK. Z-DEVD-fluorometilcetona. Z-DEVD-fluorometilcetona. inhibidor específico de la caspasa-3,

ZEA: Zearalenona

α-ZEL: alfa-zearalenol

β-ZEL: beta-zearalenol.

Resumen

La acrilamida (AA), fumitremorgina C (FTC), penitrem A (PEN A) y 3-acetildeoxinivalenol (3-ADON) son contaminantes del procesado y naturales presentes en alimentos y en el ambiente, lo que puede generar serias preocupaciones para la salud pública. La AA, clasificada como posible carcinógeno humano (Grupo 2A, IARC), se forma durante la cocción a altas temperaturas de alimentos ricos en almidón y se metaboliza en glicidamida, un compuesto capaz de causar daño en el ADN. La FTC y la PEN A son micotoxinas tóxicas producidas por hongos; la FTC es citotóxica y poco estudiada en células neuronales, mientras que la PEN A afecta los canales de potasio y la señalización gabaérgica, lo que le confiere un perfil neurotóxico y posiblemente genotóxico. El 3-ADON es una micotoxina menos estudiada que pertenece al grupo de los tricotecenos tipo B, un conjunto de compuestos sesquiterpenoides estructuralmente diversos. Es un metabolito acetilado del deoxinivalenol (DON), producido comúnmente por especies de *Fusarium* como *Fusarium graminearum* y *Fusarium culmorum*. Aunque se conoce poco acerca de su toxicidad combinada, la cual resulta particularmente relevante al considerar que los escenarios reales de exposición suelen involucrar mezclas de múltiples agentes tóxicos.

En los últimos años, ha aumentado el interés por los riesgos para la salud asociados a los contaminantes químicos en los alimentos, especialmente debido a la creciente presencia de compuestos derivados del procesamiento y de origen natural como las micotoxinas. La AA, un subproducto generado durante la cocción a altas temperaturas, y micotoxinas como la PEN A, la FTC y el 3-ADON, se detectan con frecuencia en diversos productos alimentarios y piensos. Estos compuestos han sido asociados con posibles efectos neurotóxicos, oxidativos y genotóxicos, lo cual genera una preocupación creciente en el ámbito de salud pública. En este contexto, el presente estudio tuvo como objetivo investigar los efectos toxicológicos de AA, PEN A, FTC y 3-ADON, tanto individualmente como en combinaciónes relevantes utilizando un modelo celular humano de de neuroblastoma SH-SY5Y. Para ello, se aplicó un enfoque experimental integrador centrado en la citotoxicidad, el estrés oxidativo y la genotoxicidad inducidos por estas exposiciones.

En el primer capítulo se aborda la interacción citotóxica y la alteración del ciclo celular inducidas por la exposición a AA, FTC y PEN A. En este estudio, las células SH-SY5Y fueron tratadas con AA, PEN A y FTC tanto de forma individual como en combinaciones binarias y terciarias. Las interacciones se evaluaron mediante análisis de isobologramas, mientras que los efectos sobre el ciclo celular se analizaron por citometría de flujo. Los resultados indicaron que el antagonismo fue el patrón de interacción predominante en las combinaciones binarias y terciarias. Entre los compuestos evaluados, la PEN A mostró el mayor potencial para alterar el ciclo celular, destacando por reducir la densidad celular en la fase G0/G1, lo que indica una progresión celular comprometida y posibles efectos citostáticos.

En el segundo capítulo, con el fin de comprender mejor el impacto de estos contaminantes, se analizaron diversos marcadores de estrés oxidativo. Específicamente, se evaluaron la citotoxicidad, los niveles de especies reactivas de oxígeno (ROS), la peroxidación lipídica (LPO) y el estado redox celular a través de la relación glutatión reducido/glutatión oxidado (GSH/GSSG) en células SH-SY5Y expuestas a AA, PEN A y 3-ADON

El estudio reveló que todos los compuestos administrados de forma individual aumentaron los niveles de ROS, siendo este incremento aún más marcado en las combinaciones que incluían PEN A. La peroxidación lipídica también fue más pronunciada en las combinaciones, lo que sugiere un daño acentuado en las membranas celulares. Además, se observó una disminución significativa en la relación GSH/GSSG tras la exposición a todas las mezclas, indicando un agotamiento de las defensas antioxidantes celulares. En conjunto, estos hallazgos destacan el papel central del estrés oxidativo en la toxicidad inducida por estas sustancias y subrayan la mayor peligrosidad de las exposiciones combinadas frente a las individuales, enfatizando la necesidad de considerar interacciones entre contaminantes en las evaluaciones de riesgo toxicológico.

En el tercer capítulo se evaluaron los efectos genotóxicos de AA, PEN A y 3-ADON mediante el ensayo de micronúcleos (MN) y la prueba de Ames. Los resultados del ensayo de MN mostraron un aumento significativo en el daño cromosómico para todos los tratamientos individuales, con efectos dependientes de la dosis en el caso de AA y 3-ADON. Los tratamientos combinados, particularmente AA + 3-ADON y PEN A + 3-ADON, incrementaron aún más la frecuencia de MN en comparación con los controles, lo que indica una interacción aditiva o incluso sinérgica en términos de genotoxicidad. Sin embargo, en la prueba de Ames no se detectó actividad mutagénica en ninguna de las condiciones evaluadas, lo que concuerda con la literatura previa. Estos hallazgos sugieren que, si bien estos contaminantes alimentarios no induzcan mutaciones génicas directas, sí tienen el potencial de inducir aberraciones cromosómicas, contribuyendo así a la inestabilidad genómica. Los resultados resaltan la importancia de evaluar la genotoxicidad desde una perspectiva tanto cromosómica como mutacional, especialmente en escenarios de exposición combinada.

Este estudio integral revela que la acrilamida y las micotoxinas, tanto de manera individual como combinada, representan amenazas celulares multifacéticas a través de mecanismos citotóxicos, oxidativos y genotóxicos. Los resultados refuerzan la necesidad urgente de incorporar evaluaciones de toxicidad por mezclas en las normativas de seguridad alimentaria y subrayan la importancia de fomentar investigaciones futuras que esclarezcan los mecanismos moleculares subyacentes a sus efectos combinados.

Resum

L'acrilamida (AA), fumitremorgina C (FTC), penitrem A (PEN A) i el 3-acetildeoxinivalenol (3-ADON) són contaminants de processament i naturals presents en aliments i en l'entorn, la qual cosa pot generar serioses preocupacions per a la salut pública. L'AA, classificada com a possible carcinogen humà (Grup 2A, IARC), es forma durant la cocció a altes temperatures d'aliments rics en midó i es metabolitza en glicidamida, un compost capaç de causar dany en l'ADN. La FTC i la PEN A són micotoxines tòxiques produïdes per fongs; la FTC és citotòxica i poc estudiada en cèl·lules neuronals, mentre que la PEN A afecta els canals de potassi i la senyalització gabaèrgica, la qual cosa li confereix un perfil neurotòxic i possiblement genotòxic. El 3-ADON és una micotoxina menys estudiada que pertany al grup dels tricotecens tipus B, un conjunt de compostos sesquiterpenoides estructuralment diversos. És un metabolit acetilat del deoxinivalenol (DON), produït comunament per espècies de Fusarium com Fusarium graminearum i Fusarium culmorum. Encara que es coneix poc sobre la seua toxicitat combinada, la qual resulta particularment rellevant en considerar que els escenaris reals d'exposició solen involucrar mescles de múltiples agents tòxics.

En els últims anys, ha augmentat l'interés pels riscos per a la salut associats als contaminants químics en els aliments, especialment a causa de la creixent presència de compostos derivats del processament i d'origen natural com les micotoxines. L'AA, un subproducte generat durant la cocció a altes temperatures, i micotoxines com la PEN A, la FTC i el 3-ADON, es detecten amb freqüència en diversos productes alimentaris i pinsos. Aquests compostos han estat associats amb possibles efectes neurotòxics, oxidatius i genotòxics, la qual cosa genera una preocupació creixent en l'àmbit de salut pública. En aquest context, el present estudi va tindre com a objectiu investigar els efectes toxicològics de l'AA, la PEN A, la FTC i el 3-ADON, tant individualment com en combinacions rellevants, utilitzant un model cel·lular humà de neuroblastoma SH-SY5Y. Per a això, es va aplicar un enfocament experimental integrador centrat en la citotoxicitat, l'estrés oxidatiu i la genotoxicitat induïts per aquestes exposicions.

En el primer capítol s'aborda la interacció citotòxica i l'alteració del cicle cel·lular induïdes per l'exposició a l'AA, la FTC i la PEN A. En aquest estudi, les cèl·lules SH-SY5Y van ser tractades amb l'AA, la PEN A i la FTC tant de forma individual com en combinacions binàries i terciàries. Les interaccions es van avaluar mitjançant anàlisis d'isobologrames, mentre que els efectes sobre el cicle cel·lular es van analitzar per citometria de flux. Els resultats van indicar que l'antagonisme va ser el patró d'interacció predominant en les combinacions binàries i terciàries. Entre els compostos avaluats, la PEN A va mostrar el major potencial per a alterar el cicle cel·lular, destacant per reduir la densitat cel·lular en la fase G0/G1, la qual cosa indica una progressió cel·lular compromesa i possibles efectes citostàtics.

En el segon capítol, amb la finalitat de comprendre millor l'impacte d'aquests contaminants, es van analitzar diversos marcadors d'estrés oxidatiu. Específicament, es van avaluar la citotoxicitat, els nivells d'espècies reactives d'oxigen (ROS), la peroxidació lipídica (LPO) i l'estat redox cel·lular a través de la relació glutatió reduït/glutatió oxidat (GSH/GSSG) en cèl·lules SH-SY5Y exposades a l'AA, la PEN A i el 3-ADON. L'estudi va revelar que tots els compostos administrats de forma individual van augmentar els nivells de ROS, sent aquest increment encara més marcat en les combinacions que incloïen la PEN A. La peroxidació lipídica també va ser més pronunciada en les combinacions, la qual cosa suggereix un dany accentuat en les membranes cel·lulars. A més, es va observar una disminució significativa en la relació GSH/GSSG després de l'exposició a totes les mescles, indicant un esgotament de les defenses antioxidants cel·lulars. En conjunt, aquestes troballes destaquen el paper central de l'estrés oxidatiu en la toxicitat induïda per aquestes substàncies i subratllen la major perillositat de les exposicions combinades enfront de les individuals, emfatitzant la necessitat de considerar interaccions entre contaminants en les avaluacions de risc toxicològic.

En el tercer capítol es van avaluar els efectes genotòxics de l'AA, la PEN A i el 3-ADON mitjançant l'assaig de micronuclis (MN) i la prova d'Ames. Els resultats de l'assaig de MN van mostrar un augment significatiu en el dany cromosòmic per a tots els

tractaments individuals, amb efectes dependents de la dosi en el cas de l'AA i el 3-ADON. Els tractaments combinats, particularment l'AA + 3-ADON i la PEN A + 3-ADON, van incrementar encara més la freqüència de MN en comparació amb els controls, la qual cosa indica una interacció additiva o fins i tot sinèrgica en termes de genotoxicitat. No obstant això, en la prova d'Ames no es va detectar activitat mutagènica en cap de les condicions avaluades, la qual cosa concorda amb la literatura prèvia. Aquestes troballes suggereixen que, encara que aquests contaminants alimentaris no indueixen mutacions gèniques directes, sí que tenen el potencial d'induir aberracions cromosòmiques, contribuint així a la inestabilitat genòmica. Els resultats ressalten la importància d'avaluar la genotoxicitat des d'una perspectiva tant cromosòmica com mutacional, especialment en escenaris d'exposició combinada.

Aquest estudi integral revela que l'acrilamida i les micotoxines, tant de manera individual com combinada, representen amenaces cel·lulars multifacètiques a través de mecanismes citotòxics, oxidatius i genotòxics. Els resultats reforcen la necessitat urgent d'incorporar avaluacions de toxicitat per mescles en les normatives de seguretat alimentària i subratllen la importància de fomentar investigacions futures que esclareixin els mecanismes moleculars subjacents als seus efectes combinats.

Summary

Acrylamide (AA), fumitremorgin C (FTC), penitrem A (PEN A), and 3-acetyldeoxynivalenol (3-ADON) are processed and naturally occurring contaminants found in food and the environment, raising serious public health concerns. AA, classified as a possible human carcinogen (Group 2A, IARC), is formed during the high-temperature cooking of starchy foods and is metabolized into glycidamide, a compound capable of causing DNA damage. FTC and PEN A are toxic mycotoxins produced by fungi; FTC is cytotoxic and poorly studied in neuronal cells, while PEN A affects potassium channels and GABAergic signaling, giving it a neurotoxic and potentially genotoxic profile. 3-ADON is a lesser-studied mycotoxin belonging to the type B trichothecene group a structurally diverse set of sesquiterpenoid compounds. It is an acetylated metabolite of deoxynivalenol (DON), commonly produced by Fusarium graminearum and Fusarium culmorum. Although little is known about their combined toxicity, this is particularly relevant since real-world exposure scenarios typically involve mixtures of multiple toxic agents.

In recent years, interest has grown in the health risks associated with chemical contaminants in food, especially due to the increasing presence of both processing-derived compounds and naturally occurring toxins such as mycotoxins. AA, a by-product generated during high-temperature cooking, and mycotoxins such as PEN A, FTC, and 3-ADON are frequently detected in various food products and animal feed. These compounds have been associated with potential neurotoxic, oxidative, and genotoxic effects, prompting growing concern in the field of public health. In this context, the present study aimed to investigate the toxicological effects of AA, PEN A, FTC, and 3-ADON, both individually and in relevant combinations, using the human neuroblastoma SH-SY5Y cell line as a model. An integrative experimental approach was applied, focusing on cytotoxicity, oxidative stress, and genotoxicity induced by these exposures.

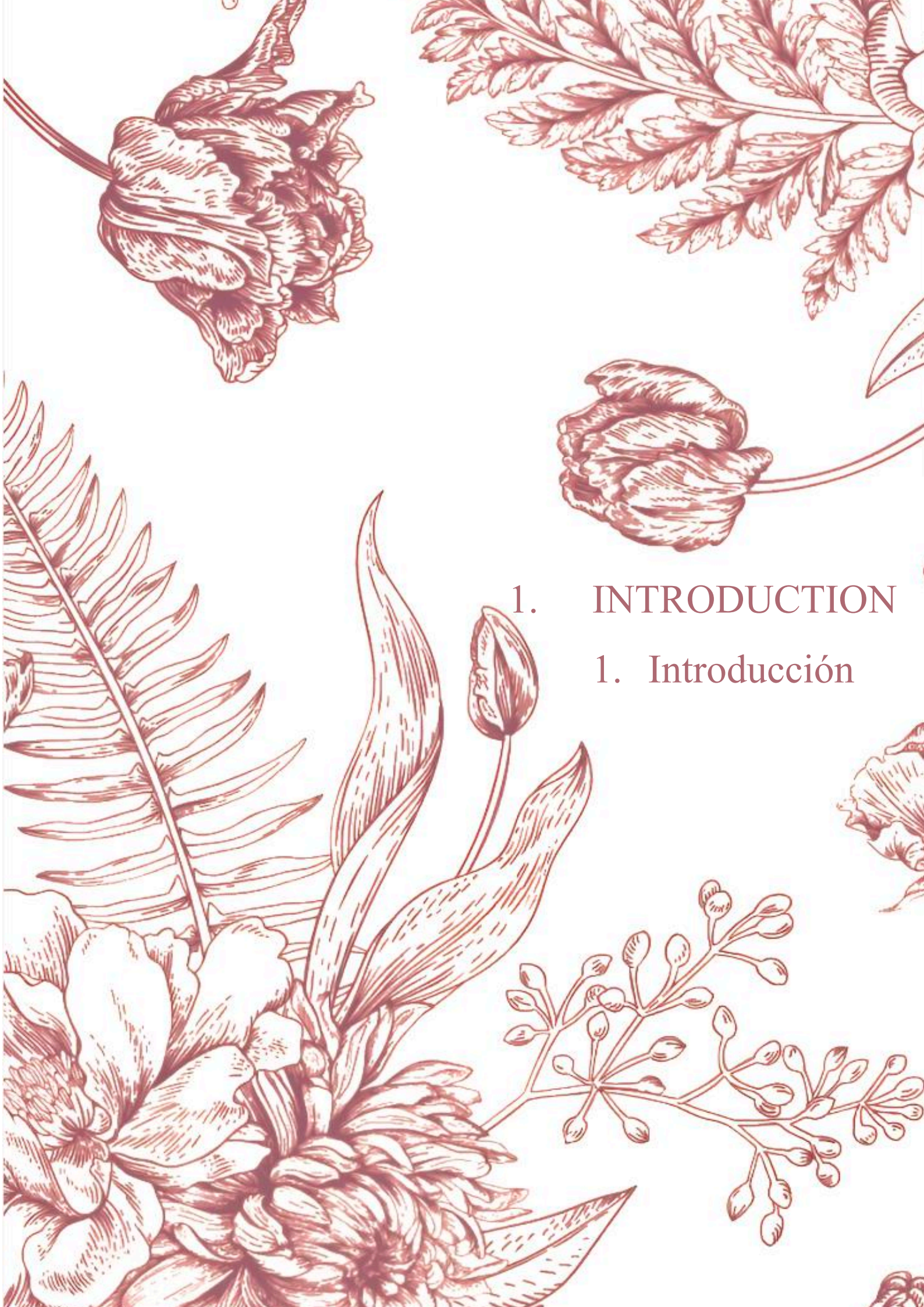
The first chapter addresses cytotoxic interactions and cell cycle alterations induced by exposure to AA, FTC, and PEN A. In this study, SH-SY5Y cells were treated with AA, PEN A, and FTC, both individually and in binary and tertiary combinations. Interactions were evaluated through isobologram analysis, while cell cycle effects were assessed via flow cytometry. The results indicated that antagonism was the predominant interaction pattern in both binary and tertiary combinations. Among the compounds evaluated, PEN A showed the greatest potential to alter the cell cycle, particularly by reducing cell density in the G0/G1 phase, indicating impaired cell cycle progression and possible cytostatic effects.

In the second chapter, to better understand the impact of these contaminants, various oxidative stress markers were analyzed. Specifically, cytotoxicity, reactive oxygen species (ROS) levels, lipid peroxidation (LPO), and the cellular redox state via the reduced/oxidized glutathione ratio (GSH/GSSG) were evaluated in SH-SY5Y cells exposed to AA, PEN A, and 3-ADON. The study revealed that all individually administered compounds increased ROS levels, with this increase being more pronounced in combinations that included PEN A. Lipid peroxidation was also more severe in the combinations, suggesting greater damage to cell membranes. In addition, a significant decrease in the GSH/GSSG ratio was observed after exposure to all mixtures, indicating a depletion of cellular antioxidant defenses. Taken together, these findings highlight the central role of oxidative stress in the toxicity induced by these substances and underscore the greater risk posed by combined exposures compared to individual ones, emphasizing the need to consider contaminant interactions in toxicological risk assessments.

The third chapter evaluated the genotoxic effects of AA, PEN A, and 3-ADON using the micronucleus (MN) assay and the Ames test. The MN assay results showed a significant increase in chromosomal damage for all individual treatments, with dose-dependent effects observed for AA and 3-ADON. Combined treatments—particularly AA + 3-ADON and PEN A + 3-ADON—further increased MN frequency compared to controls, indicating an additive or even synergistic interaction in terms of genotoxicity.

However, the Ames test did not detect mutagenic activity under any of the conditions tested, which aligns with previous literature. These findings suggest that, while these food contaminants may not induce direct gene mutations, they do have the potential to cause chromosomal aberrations, contributing to genomic instability. The results underscore the importance of evaluating genotoxicity from both chromosomal and mutational perspectives, especially in scenarios involving combined exposure.

This comprehensive study reveals that acrylamide and mycotoxins, both individually and in combination, represent multifaceted cellular threats through cytotoxic, oxidative, and genotoxic mechanisms. The findings reinforce the urgent need to incorporate mixture toxicity evaluations into food safety regulations and highlight the importance of promoting future research to elucidate the molecular mechanisms underlying their combined effects.



1. INTRODUCTION

1. Introducción

1. INTRODUCCIÓN

La seguridad alimentaria se ve comprometida por la presencia de múltiples contaminantes químicos que pueden generarse durante el procesado de los alimentos o estar presentes de forma natural en las materias primas. Entre ellos, la acrilamida (AA) y diversas micotoxinas representan una preocupación creciente por sus efectos tóxicos y potencial neurotoxicidad. La acrilamida es un contaminante de procesado que se forma principalmente en alimentos ricos en almidón durante tratamientos térmicos como el horneado o la fritura, a través de la reacción de Maillard. Por otro lado, las micotoxinas como el Penitrem A (PEN A), el 3-acetildeoxinivalenol (3-ADON) y el Fumitremorgin C (FTC) son compuestos naturales producidos por hongos como *Penicillium* y *Fusarium*, que pueden contaminar cereales y otros productos agrícolas. Estas sustancias, aunque de distinta naturaleza y origen, pueden coexistir en los mismos alimentos y presentar efectos adversos combinados sobre la salud humana. La exposición conjunta a contaminantes de procesado y micotoxinas representa un escenario realista en la dieta cotidiana, y su evaluación toxicológica es fundamental para comprender sus mecanismos de acción y posibles riesgos sinérgicos. A continuación, se describen las generalidades de la acrilamida y de las micotoxinas, abordando sus orígenes, vías de formación y relevancia toxicológica.

1.1. Acrilamida y micotoxinas: generalidades

El proceso de horneado de alimentos es el subproceso más importante responsable de las principales propiedades químicas, físicas y sensoriales del producto final, así como del desarrollo de compuestos bioactivos y antioxidantes (Anese 2015; Guenther et al. 2007; Soares et al., 2014). Sin embargo, este proceso térmico induce la formación de componentes tóxicos no deseados, incluida la acrilamida (AA), debido a la reacción de Maillard que ocurre a alta temperatura en alimentos ricos en azúcares reductores y aminoácidos o proteínas (Anese 2015; Rannou et al. 2016; Taeymans et al. 2004; Tareke et al. 2002). En detalle, la reacción de Maillard implica tres pasos principales: (i) condensación de grupos amino libres (como la asparagina) con azúcares reductores (glucosa y fructosa) para formar acroleína; ii) Degradación de los aminoácidos a

aldehídos y amoníaco; y (iii) compuestos nitrogenados marrones que se combinan con ácido acrílico para formar AA (Figura 1) (Xiang et al., 2021).

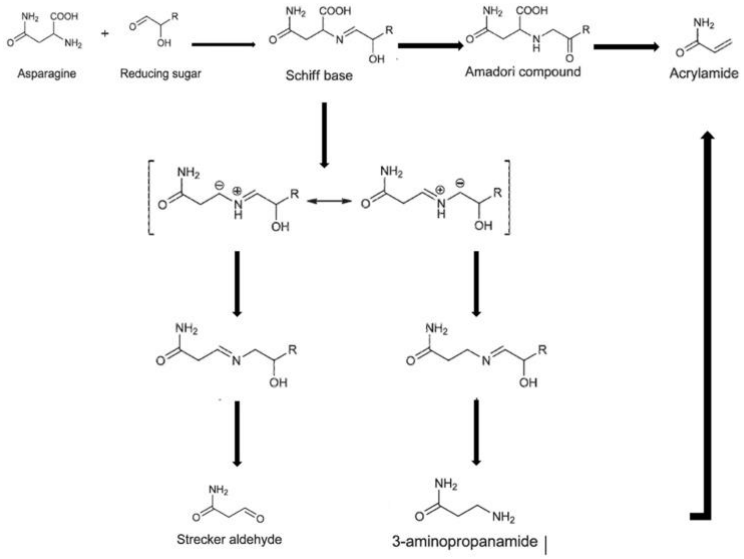


Figura 1. *Mecanismo propuesto de formación de Acrilamida asociado a la reacción de Maillard.*

El compuesto AA es un compuesto cristalino incoloro e inodoro, altamente reactivo y soluble en agua con un bajo peso molecular de 71,08 kDa. El AA se utiliza en muchas áreas industriales diferentes, como el tratamiento de aguas y la producción de papel, telas o cosméticos, y se emplea ampliamente en laboratorios para cromatografía en gel. El uso generalizado de AA provoca un alto nivel de exposición ocupacional por inhalación y contacto con la piel (Tepe & Çebi, 2019). Además, en 2002, investigadores suecos informaron que varios alimentos ricos en carbohidratos tratados térmicamente contenían niveles significativamente más altos de AA que otros carcinógenos alimentarios conocidos (Tareke et al., 2002). Sin embargo, varios factores pueden influir en el nivel de formación de AA en los alimentos, como el tipo de materias primas, la composición del producto, el pH y la humedad. Además, los niveles más altos de AA se detectan en los alimentos derivados de procesos térmicos que implican la fritura y el dorado de las patatas, los granos de cacao y el tostado del café, así como en el horneado

de cereales y pasteles (Michalak et al. 2014). Es interesante destacar que el AA fue clasificado como un compuesto "posiblemente cancerígeno para el ser humano" por la Agencia Internacional para la Investigación del Cáncer y clasificado en el Grupo 2A. Asimismo, la Comisión Europea (2017) (IARC, 1994) clasifica al AA como una sustancia carcinógena y mutágena de Clase 1B y de Clase 2 por su toxicidad reproductiva, mientras que la Agencia Europea de Sustancias y Mezclas Químicas (2022) ha incluido al AA en la lista de sustancias extremadamente preocupantes. Por lo tanto, es de gran interés, y se requieren necesariamente más estudios de la toxicidad de los AA para evaluar el riesgo potencial para la salud humana.

(El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011)(El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011)(El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011)(El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011)(El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011)(El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011)Las características toxicodinámicas asociadas con el AA se basan en una rápida absorción en la sangre y una amplia distribución a los tejidos debido a sus propiedades de bajo peso molecular y alta solubilidad (Exon, 2006). El AA es metabolizado principalmente en el hígado y el cerebro por la enzima glutatión-S-transferasa, formando N-acetil-S-(3-amino-3-oxipropil)-cisteína (Mottram y Friedman, 2005). Además, el AA puede ser convertido por el citocromo P450 2E1 (CYP2E1) en glicidamida (GA), un epóxido más reactivo que puede reaccionar con la hemoglobina y el ADN (Ghanayem et al., 2005; Nowak et al., 2020). Por lo tanto, el GA es responsable de los efectos mutagénicos y cancerígenos del AA in vivo. Se ha demostrado que el AA induce efectos secundarios sistémicos en animales, mientras que se ha detectado toxicidad cerebral en humanos (Kopańska et al., 2022; LoPachin y Gavin, 2012). También se ha informado de que la neurotoxicidad del AA se asocia con el desequilibrio entre la oxidación y la función antioxidante provocado por la peroxidación lipídica y un aumento de las especies reactivas de oxígeno (ROS) intracelulares (LoPachin et al., 2005).

Por otro lado, las micotoxinas son metabolitos secundarios tóxicos producidos por varias (Eisenbrand, 2020a)especies de hongos *Aspergillus, Penicillium, Fusarium* y *Trichoderma* que son tóxicos peligrosos para la salud que con frecuencia están presentes en varios productos agrícolas, incluidos granos, nueces y especias (Tola y Kebede, 2016). Estas toxinas plantean riesgos significativos para la salud de los seres humanos y los animales cuando se consumen (Zain, 2011). Comprender los efectos de las micotoxinas en diferentes tipos de células es crucial para evaluar su impacto toxicológico. El estudio de las micotoxinas (El-Sayed et al., 2022; Abudayyak et al., 2022; Doi & Uetsuka, 2011) *in vitro* utilizando células SH-SY5Y permite a los investigadores evaluar sus posibles efectos neurotóxicos y dilucidar los mecanismos subyacentes de toxicidad. Estos estudios implican exponer las células a diversas concentraciones de micotoxinas específicas, como la ocratoxina A (OTA) o las fumonisinas, y evaluar su impacto en la viabilidad celular, la morfología, la proliferación, la apoptosis, el estrés oxidativo, la función de los neurotransmisores y otros criterios de valoración relevantes (Abudayyak et al., 2022; Doi y Uetsuka, 2011). Asimismo, la Comisión Europea ha notificado más de 400 alertas por contaminación fúngica en alimentos y piensos, siendo la OTA una de las toxinas más notificadas, representando el 10% de las notificaciones (RASFF, 2020). Hoy en día, existe un fuerte esfuerzo para estudiar la combinación de compuestos que pueden ocurrir en la misma matriz alimentaria, ambiente, o que se ingieren diariamente a través de la dieta, especialmente en lo que respecta a los efectos que pueden causar los compuestos mezclados. La neurotoxicidad de los AA y las micotoxinas se revisa en varios modelos *in vitro*, incluidos los modelos de línea celular de neuroblastoma humano indiferenciado y diferenciado (SH-SY5Y). Las células SH-SY5Y se utilizan en la investigación toxicológica para evaluar la diferenciación, el metabolismo y la función neuronal, así como en los procesos neuroadaptativos, neurodegenerativos y de neuroprotección (Xie et al., 2010). Las células humanas SH-SY5Y son un subclon de SK-NSH, que, una vez estimuladas con ácido retinoico (AR), se diferencia en células dopaminérgicas similares a las neuronas, adquiriendo muchas propiedades bioquímicas y funcionales de las neuronas (Kalinovskii et al., 2022; Singh et al., 2007).

Además, un nuevo estudio establece los efectos combinados de estos compuestos en las células SH-SY5Y, haciendo hincapié en la importancia de recopilar datos sobre AA y micotoxinas de forma individual para comprender sus mecanismos de acción (Bridgeman et al., 2023). Además, el interés en los efectos tóxicos compartidos documentados tanto en la acrilamida (AA) como en múltiples micotoxinas no se debe solo a su presencia en nuestras dietas, sino que también es atribuible a la posible combinación de estos efectos, lo que plantea importantes implicaciones para la salud humana, especialmente en lo que respecta a los impactos neuronales. Por lo tanto, este trabajo tuvo como objetivo evaluar los efectos de AA y micotoxinas, así como su mecanismo de acción (MoA) en la línea celular de neuroblastoma humano SH-SY5Y.

A pesar de la amplia investigación sobre micotoxinas bien conocidas, como las aflatoxinas (AF), las ocratoxinas (OTA) y el deoxinivalenol (DON), siguen existiendo otros grupos de micotoxinas menos estudiados que son igualmente preocupantes. Entre ellos se encuentran las tremortoxinas, como el penitrem A, que son producidas por ciertas especies de *Penicillium* y son conocidas por sus efectos neurotóxicos, causando síntomas como temblores y convulsiones en los animales. Además, los metabolitos de las micotoxinas, como el 3-acetildeoxinivalenol (3-ADON), están emergiendo como toxinas importantes, pero poco investigadas. Estos metabolitos pueden presentar diferentes perfiles de toxicidad en comparación con sus compuestos originales, lo que podría plantear nuevos riesgos para la salud humana y animal. La comprensión limitada de estas micotoxinas menos conocidas pone de manifiesto la necesidad de realizar más investigaciones para evaluar plenamente su prevalencia ambiental, sus mecanismos de toxicidad y sus posibles impactos en la salud.

1.2. Penitrem A (PEN A)

Un tipo de micotoxinas menos estudiado son las micotoxinas tremorgénicas, que son metabolitos fúngicos secundarios que provocan temblores intermitentes o sostenidos en las especies de vertebrados (Evans y Gupta, 2018).

PEN A (Figura 2b) es una potente neurotoxina producida por *Penicillium spp.,* especialmente *Penicillium crustosum*, conocido por causar un síndrome tremorgénico en vertebrados, caracterizado por temblores sostenidos y, en dosis altas, convulsiones y muerte en animales de laboratorio y de granja (Wilson, 1971; Arp y Richard, 1981; Peterson y Penny, 1982; Shreeve et al., 1983; Hocking et al., 1988; Breton et al., 1998; Cavanagh et al., 1998). Esta micotoxina se encuentra comúnmente en alimentos y piensos contaminados, como nueces, nueces, cereales, carne, queso y otros productos infectados por hongos *Penicillium* (Richard et al., 1981; Boysen et al., 2002; Young et al., 2003; Munday et al., 2008). Los perros, así como el ganado vacuno, ovino y equino, pueden envenenarse al consumir alimentos contaminados con *Penicillium spp.* que producen PEN A (Cavanagh et al., 1998; Hocking et al., 1988; Boysen et al., 2002; Walter, 2002; Young et al., 2003). Se ha demostrado que PEN A interrumpe la liberación de neurotransmisores, en particular aumentando la liberación espontánea de glutamato, aspartato y GABA, que son neurotransmisores excitadores asociados con las células de Purkinje. Esta micotoxina se dirige a las células cerebelosas de Purkinje, causando una degeneración y necrosis generalizadas en esta región del cerebro, sin afectar a otras áreas del cerebro (Catovic et al., 1975; Cavanagh et al., 1998). Además, PEN A atraviesa la barrera hematoencefálica, lo que refuerza la teoría de que es el principal compuesto responsable de sus efectos neurotóxicos. Además, se ha descubierto que el PEN A induce la producción de especies reactivas de oxígeno (ROS), lo que sugiere que el estrés oxidativo puede desempeñar un papel en su toxicidad. Los efectos neurotóxicos del PEN A, en particular su síndrome tremorgénico, han suscitado preocupación por los riesgos potenciales para la salud humana, aunque los casos de envenenamiento humano siguen siendo limitados. De hecho, algunos casos reportados vinculan la ingestión de *Penicillium crustosum* con síndromes de temblores genéticos en humanos, lo que genera preocupaciones de que tales incidentes puedan estar subestimados y destaca la necesidad de más investigación sobre los riesgos para la salud que plantean los mohos tremorgénicos (Lewis et al., 2005).

Los efectos neurotóxicos de PEN A se atribuyen principalmente a su capacidad para inhibir los canales de K^+ regulados por Ca^{2+} *in vitro,* lo que provoca interrupciones en

la secreción de neurotransmisores, incluidos glutamato, aspartato y GABA, en los sinaptosomas cerebrocorticales de ratas y ovejas. Además, la investigación sugiere que el PEN A tiene la capacidad de cruzar la barrera hematoencefálica (BBB) y no sus metabolitos, lo que refuerza la noción de que el PEN A en sí mismo es el impulsor clave de su actividad neurotóxica. Además, los estudios han demostrado que PEN A induce la producción de especies reactivas de oxígeno (ROS) en las neuronas granulares cerebelosas primarias de ratas, lo que muestra un posible mecanismo de toxicidad relacionado con el estrés oxidativo. Teniendo en cuenta estos hallazgos, es esencial una comprensión más profunda del impacto de PEN A en la salud humana, particularmente en relación con su papel en la neurotoxicidad y sus riesgos potenciales a través de la contaminación de los alimentos (Norris et al., 1980; Moldes-Anaya et al., 2009; Berntsen et al., 2017a).

1.3. 3-acetildeoxinivalenol (3-ADON)

El 3-acetildeoxinivalenol (3-ADON) es una micotoxina tricotecénica de tipo B, menos estudiada que otras, y producida por varias especies del género *Fusarium*, en particular *Fusarium graminearum* y *Fusarium culmorum* (Pasquali et al., 2016). Se trata de un metabolito acetilado del deoxinivalenol (DON), una micotoxina comúnmente presente en alimentos y piensos elaborados a base de cereales como trigo, cebada y maíz. Químicamente, el 3-ADON pertenece al grupo de los tricotecenos de tipo B, compuestos sesquiterpenoides que presentan una considerable diversidad estructural (Figura 2c).

Las cepas productoras de 3-ADON son especialmente prevalentes en regiones de Europa y Asia (De Boevre et al., 2012; Juan-García et al., 2015a). Esta micotoxina suele encontrarse como cocontaminante junto con el DON y otros tricotecenos, lo que plantea inquietudes respecto a sus posibles efectos toxicológicos, en particular cuando se presentan en mezclas (Wu y Wang, 2016).

Estructuralmente, el 3-ADON difiere del DON por la presencia de un grupo acetilo en la posición C-3, que puede influir en su absorción, metabolismo y toxicidad. Si bien el DON está bien documentado por su capacidad para inhibir la síntesis de proteínas e inducir estrés oxidativo, el perfil toxicológico del 3-ADON sigue siendo menos explorado. Sin embargo, los estudios sugieren que el 3-ADON puede compartir mecanismos similares de toxicidad, incluido el estrés ribotóxico, las respuestas inflamatorias y la alteración de la integridad de la barrera intestinal (Murtaza et al., 2024;Pintón y Oswald, 2014; Riahi et al., 2021).

Las agencias reguladoras, como la Autoridad Europea de Seguridad Alimentaria (EFSA) y la Administración de Alimentos y Medicamentos de EEUU (FDA), han establecido pautas para la exposición al DON, pero los datos sobre el 3-ADON aún son limitados. Debido a su frecuente presencia en alimentos y piensos, su conversión metabólica en DON en el organismo y sus posibles efectos tóxicos es necesario seguir investigando para evaluar su impacto en la salud humana y animal.(Wu y Wang, 2016).

Este creciente interés en el 3-ADON ha llevado a aumentar los esfuerzos para evaluar su toxicocinética, citotoxicidad y genotoxicidad. Los estudios centrados en su capacidad para inducir citotoxicidad, estrés oxidativo y daño en el ADN, incluida la formación de micronúcleos y el potencial mutagénico en sistemas bacterianos como la prueba de Ames, son cruciales para comprender los riesgos asociados con la exposición. Dado que las mezclas de micotoxinas se encuentran comúnmente en fuentes de alimentos contaminados, la evaluación de los efectos combinatorios de 3-ADON con otras micotoxinas también es esencial para una evaluación de riesgos integral.

1.4. Fumitremorgina C (FTC)

La Fumitremorgina C (FTC) (Figura 2d) no mostró citotoxicidad por sí misma. Sin embargo, se descubrió que también puede revertir la resistencia a la doxorrubicina, bisantreno y topotecán, lo cual es un indicio característico de resistencia mediada por transportadores de múltiples fármacos. De hecho, otro estudio identificó que la FTC

actúa sobre el transportador de resistencia múltiple ABCG2, un miembro de la superfamilia de casetes de unión a ATP (ABCG) (Calcagno et al., 2008).

La FTC fue el primer compuesto natural aislado que inhibe específicamente la proteína BCRP (Breast Cancer Resistance Protein), y fue hallada en *Aspergillus fumigatus* (Rabindran et al., 1998). Posteriormente, se encontró que este compuesto presentaba efectos neurotóxicos en líneas celulares humanas (Allen et al., 2002).

Estos hallazgos han generado un creciente interés por explorar más a fondo su impacto en la salud humana, particularmente en relación con su capacidad para dirigirse a transportadores de resistencia a múltiples fármacos y su potencial papel en la modulación de la resistencia a medicamentos en contextos clínicos. No obstante, se requiere más investigación para dilucidar completamente el espectro de efectos biológicos de la FTC, incluidos sus mecanismos de neurotoxicidad.

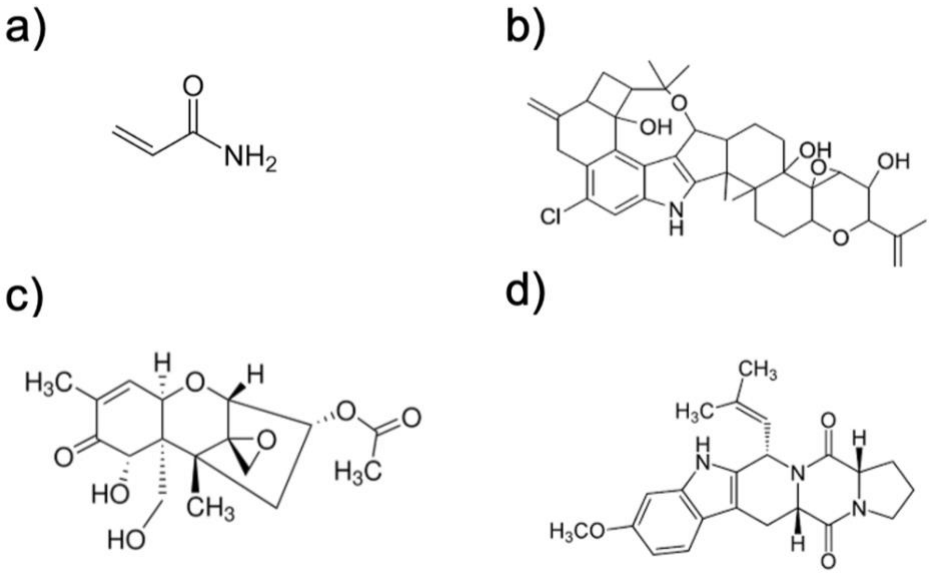


Figura 2. Estructuras químicas de los compuestos de estudio: (a) AA, (b) PEN A, (c) 3-ADON, and (d) FTC.

A continuación, se presentan los resultados (de toxicidad) relacionados con la acrilamida (AA) y las micotoxinas que pueden coexistir en alimentos y productos

alimenticios a base de cereales. Esta sección se ha organizado en las siguientes subsecciones: citotoxicidad, apoptosis, estrés oxidativo, degeneración de la red, vías de señalización, expresión de proteínas, y el papel de compuestos naturales en la mitigación de los efectos adversos inducidos por la AA.

1.5. Citotoxicidad

1.5.1. Acrilamida (AA)

Las células no viables o muertas suelen asociarse con la pérdida de la integridad de la membrana plasmática, lo cual ocurre debido a alteraciones en el transporte de moléculas hacia el interior o exterior de la célula a través de membranas que se han vuelto permeables (Riss et al., 2019). Los ensayos de citotoxicidad son herramientas clave para evaluar la toxicidad de compuestos químicos en función de la dosis y el tiempo de exposición, así como para determinar la reversibilidad de los efectos y su impacto en el ciclo celular. Existen diversos métodos para evaluar la citotoxicidad, que varían en sus enfoques: algunos se basan en observaciones morfológicas visuales (como las pruebas de elución y de contacto directo o indirecto), mientras que otros utilizan técnicas de análisis más rápidas y cuantitativas, como métodos colorimétricos o de recuento celular. Entre estos últimos se incluyen la exclusión con azul de tripano, la absorción de yoduro de propidio y el ensayo con bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-2H-tetrazolio (MTT), entre otros (Gupta et al., 2022).

En este sentido, para conocer la citotoxicidad de AA a nivel neuronal, varios autores investigaron la citotoxicidad mediante ensayo MTT en células SH-SY5Y cuando se incubaron con diferentes concentraciones de AA durante 24 h (Yan et al., 2019). Se detectaron reducciones en la viabilidad celular del 15% al 40% en las células SH-SY5Y tratadas con AA (de 2 a 5 mM) de manera dosis-dependiente (Yan et al., 2019; Yan et al., 2022; Song et al., 2017). Además, se detectaron cambios en la forma de las células después de la exposición a 2,5 o 5 mM de AA, durante 24 h, ya que la mayoría de las células se encogieron y el cuerpo celular se volvió redondo (Yan et al., 2019). En la misma línea de investigación pero un paso adelante, el mismo grupo de investigadores estudió el efecto protector de la curcumina sobre la citotoxicidad inducida por AA para

la cual las células SH-SY5Y tratadas con AA de 2,5 a 10 mM obtuvieron una disminución en el número de células vivas del 14% al 61% con respecto al control mediante un ensayo CCK8 (Yan et al., 2022), mientras que el tratamiento de las células SH-SY5Y con 100 μM de ácido α-lipoico (LA) suprimió la inducción de la apoptosis AA y la pérdida de viabilidad celular.

De manera similar, al comparar la citotoxicidad inducida por acrilamida (AA) en células SH-SY5Y y en una línea celular de glioblastoma humano (U-1240 MG), se observó una respuesta diferencial dependiente del tiempo y la dosis. En un estudio, tras el tratamiento con concentraciones de AA entre 0,1 y 2 mM durante un periodo de hasta 72 horas, la viabilidad de las células SH-SY5Y se redujo significativamente, alcanzando una disminución del 40 % a una concentración de 2 mM. En contraste, las células U-1240 MG mostraron una reducción mucho mayor en la viabilidad, con una disminución del 92 % a la misma concentración de AA (Chen y Chou, 2015).

Resultados consistentes con la disminución de viabilidad en células SH-SY5Y también fueron reportados a concentraciones más bajas (100 μM) tras 72 horas de exposición (Attoff et al., 2016). Además del ensayo MTT, que es uno de los métodos más comunes para evaluar la citotoxicidad, se emplearon otras pruebas, como el ensayo de formación de colonias (SF), la exclusión de azul de tripano y la liberación de lactato deshidrogenasa (LDH), para detectar los efectos citotóxicos de la AA (Komoike y Matsuoka, 2016; Chen et al., 2019).

En particular, el ensayo SF reveló que altas concentraciones de AA (10 mM) inducen un daño celular severo y muerte celular en exposiciones tan cortas como 8 horas (Komoike y Matsuoka, 2016). Por su parte, los ensayos de exclusión con azul de tripano y liberación de LDH también mostraron una reducción significativa de la viabilidad celular (38 %) a las 6 horas de exposición a 10 mM de AA (Chen et al., 2019).

Otro estudio realizado por Okuno et al. (2006), también en células SH-SY5Y, mostró que la citotoxicidad de AA dependía de la dosis (0,5-5 mM) y el tiempo (1-24 h), por el hecho de que la exclusión de azul de tripano disminuyó y la fuga de LDH aumentó (Okuno et al., 2006). El ensayo WST-8 es otro ensayo para la viabilidad celular y se comparó con la fuga de LDH en SH-SY5Y expuesto a un rango de concentración de 1-5 mM AA (Sumizawa, & Igisu, 2007); los resultados revelaron una disminución de WST-8 y un aumento de LDH según la dosis AA, pero ayudaron a confirmar los resultados anteriores (Sumizawa e Igisu, 2007). Por otro lado, la posibilidad de que las células SH-SY5Y se diferencien como neuronas permitió a los investigadores estudiar el efecto de AA mediante el ensayo LDH, revelando que era en gran medida no citotóxico a 1 h de exposición, excepto a dosis más altas (10 mM); esto fue similar a los resultados cuando se probaron células SH-SY5Y indiferenciadas (Hartley et al., 1997). Además, Frimat et al. (2010) demostraron a través del ensayo de viabilidad con CellTiter-Blue que una concentración de AA de 5 mM alcanzó una concentración de inhibición del 50% (IC_{50}) para las células SH-SY5Y, mientras que 0,26 mM provocó una reducción del 20% en la formación de la red equivalente al control a las 24 h de exposición En otro estudio, la citotoxicidad basal fue determinada mediante la cuantificación del contenido total de proteínas celulares. Los valores de proteína total asociados con las subunidades TP50 y TP20 (1,34 mmol/L y 0,61 mmol/L, respectivamente) reflejaron niveles de citotoxicidad grave y moderada en células SH-SY5Y expuestas a AA (Nordin-Andersson et al., 2003). Sin embargo, otros investigadores observaron que concentraciones de AA entre 0,5 y 2,0 mM no produjeron efectos citotóxicos significativos en un periodo de 24 horas, evaluados mediante microscopía de fluorescencia. No obstante, concentraciones superiores a 4 mM provocaron una pérdida significativa de neuronas HuD-positivas (reducción del 58 % con respecto al control) y una disminución del número de axones en un 21 % (Lourenssen et al., 2009).

De manera similar, utilizando el ensayo de viabilidad celular basado en fluorescencia Calcein-AM, se encontró que 1 mM de AA no causó toxicidad significativa a las 48 horas en células SH-SY5Y ni en la línea celular de carcinoma embrionario murino P19.

En contraste, esta misma concentración sí resultó citotóxica para la línea celular PC12, derivada de feocromocitoma de médula suprarrenal de rata (Popova et al., 2017). Otro estudio, mediante microscopía de fluorescencia con un kit de tinción de células vivas/muertas II, evaluó la citotoxicidad tras 24 horas de exposición a diferentes concentraciones de AA (0,01, 0,28 y 7 mM) en células SH-SY5Y. Los resultados indicaron una disminución significativa en la viabilidad celular a concentraciones de 0,28 mM y 7 mM (Ban et al., 2021).

Con el fin de obtener resultados más precisos, se estudió la citotoxicidad de AA en células SH-SY5Y mediante ensayo MTS y se evaluó la toxicidad después de 24 h de incubación (Hallier-Vanuxeem et al., 2009). La cantidad obtenida en la concentración de abluminal después de 1 h también se midió en el modelo de barrera hematoencefálica (BBB), para finalmente comparar los resultados de citotoxicidad entre AA ensayados directamente en SH-SY5Y después de 24 h con el AA obtenido después de pasar por un modelo in vitro de BBB (4 d/24 w) durante 1 h. Se ha demostrado que la acrilamida (AA) es citotóxica a una concentración de 100 µM en células SH-SY5Y tras 24 horas de exposición. Sin embargo, esta citotoxicidad no se observó con la concentración de AA en el compartimento abluminal tras su paso a través del modelo de barrera hematoencefálica (BBB), lo que sugiere una posible atenuación del efecto tóxico después del transporte (Hallier-Vanuxeem et al., 2009).

1.5.2. Micotoxinas

En la siguiente sección se abordará la capacidad de alteracion de la viabilidad celular, concretamente de la citotoxicidad de varias micotoxinas relevantes en seguridad alimentaria. Se analizarán específicamente los efectos de BEA, DON, ENN, FB1, OTA, T-2 y ZEA.

1.5.2.1. Beauvericina (BEA)

Los estudios sobre el impacto de BEA en las células SH-SY5Y han mostrado resultados variados, principalmente a través de la utilización del ensayo MTT. Se demostró que la

viabilidad se reducía en un 50% durante una exposición de 72 h a 2,5 µM, y algunos estudios citan una disminución del 43% en la viabilidad a la concentración más alta ensayada (2,5 µM) después de 48 h de exposición (Juan et al., 2020; Juan-García et al., 2021; Agahi et al., 2020; Agahi et al., 2022). Asimismo, se han documentado distintos valores de IC_{50} en función del tiempo de exposición, con concentraciones de 1,9 µM a las 6 horas, 1,7 µM a las 24 horas y 1,5 µM a las 48 horas (Pérez-Fuentes et al., 2021; Montesano et al., 2020). Además, se observó que una concentración de 1 µM de BEA inducía liberación de lactato deshidrogenasa (LDH), lo que respalda los resultados obtenidos mediante el ensayo MTT y sugiere un daño celular asociado (Montesano et al., 2020).

1.5.2.2. Deoxinivalenol (DON)

En cuanto a los efectos del DON en células SH-SY5Y, se utilizaron ensayos MTT en ambos estudios, aunque con marcadas diferencias en las concentraciones evaluadas y los resultados obtenidos. Pérez-Fuentes et al. (2021) reportaron un valor de IC_{50} de 2,25 µM tras 24 horas de exposición, mientras que Kalagatur et al. (2021) encontraron un IC_{50} significativamente mayor, de 120 µM, al mismo tiempo de exposición. Además, mientras que Pérez-Fuentes et al. (2021) no observaron un aumento en los niveles extracelulares de LDH, Kalagatur et al. (2021) reportaron una liberación de LDH directamente proporcional a la reducción de la viabilidad celular observada en el ensayo MTT, lo que sugiere un mayor daño en la membrana celular.

1.5.2.3. Enniatina A and Enniatina B (ENN A and ENN B)

Seis horas de exposición a ENN A dieron lugar a una caída del 41,0 ± 8,5% en la viabilidad celular a 2,5 µM y del 94,3 ± 1,6% a 5 µM, respectivamente, con inhibición total a 10 µM. Después de la incubación durante 24 y 48 h, la viabilidad celular a 2,5 y 5 µM mostró una disminución superior al 74,5%, y a la dosis más alta probada, se observó una reducción completa una vez más. En cuanto a la ENN B, la viabilidad celular no alcanzó la inhibición total a la dosis más alta a las 6 h (Pérez-Fuentes et al., 2021). Sin embargo, a las 24 h, ENN B presentó un IC_{50} de 0,43 µM. Además, ENN A

aumentó la liberación de LDH a 5 y 10 µM a las 24 y 48 h. Después del tratamiento durante 24 h, ENN B aumentó la liberación de LDH en aproximadamente un 20% a valores superiores a 0,25 µM. Después de 6 h de incubación, a 5 y 10 µM, ENN A mostró diferencias sustanciales con respecto a las células control (79.0 ± 8.9% y 67.7 ± 14.5%, respectivamente) (Pérez-Fuentes et al., 2021).

1.5.2.4. Fumonisina B1 (FB1)

En cuanto a los efectos de FB1, varios estudios investigaron el potencial tóxico de esta micotoxina en las células SH-SY5Y. En total, 100 µM de FB1 disminuyeron la viabilidad celular de las células SH-SY5Y después de la incubación durante 48-144 h (Stockmann-Juvala et al., 2009). En la misma línea, pero con tiempos de exposición más bajos, después de 48 h de incubación, FB1 redujo la viabilidad celular a la concentración más alta (30 µM), y pareció promover la proliferación celular a las dosis más bajas (0,1 µM) después de 24 y 48 h. Además, no se encontró IC_{50} y en el ensayo de LDH no se mostraron efectos citotóxicos tras el tratamiento con FB1 (Montesano et al., 2020). Del mismo modo, en otro estudio, se comparó el tratamiento de 50 µM de FB1 con el control, y la LDH se liberó a las 12 h, pero esta liberación disminuyó a las 24 h y a las 48 h de tratamiento y no hubo diferencia con el control (Paul et al., 2021). A continuación, Domijan et al. (2011) investigaron cómo FB1 (en concentraciones que oscilaban entre 0,5 y 200 M) afectaba a la viabilidad de los cultivos celulares tras 24 h de exposición. Ninguna de las concentraciones de FB1 utilizadas en el experimento causó la muerte celular en las células de neuroblastoma. Las células de neuroblastoma tratadas durante 24 h con la dosis máxima de FB1 (200 µM) mostraron una supervivencia celular del 98,0 ± 1,85%.

1.5.2.5. Ocratoxina A (OTA)

En lo que concierne a la viabilidad de las células SH-SY5Y tratadas con OTA, los valores de IC_{50} observados a las 24 h y 48 h fueron de 9,1 µM y 5,8 µM, respectivamente (Penalva-Olcina et al., 2022). Se observó una disminución significativa de la viabilidad más allá de concentraciones superiores a 3,12 µM o 6,25 µM durante 24 h y 48 h, lo

que resultó en una disminución del 74% al 25% y del 80% al 49% a las 24 h y 48 h, respectivamente (Penalva-Olcina et al., 2022). De manera similar, se observaron disminuciones menos dramáticas en la actividad mitocondrial bajo diferentes concentraciones de tratamiento con OTA (74 ± 12 y 74 ± 44% de control a 10 y 100 µM de OTA, respectivamente) a las 24 h, y las actividades de LDH de OTA a 1, 10 o 100 µM fueron de 128 ± 1,125 ± 2, y 200 ± 1% del control no tratado (Yoon et al., 2009).

1.5.2.6. T-2 Toxin

En cuanto a la exposición de las células SH-SY5Y a la toxina T-2, esto resultó en una disminución significativa de la viabilidad celular, con porcentajes de 81,9%, 40,8% y 35,5% observados para las células expuestas a 5, 10 y 20 ng/mL de la toxina durante 48 h, respectivamente. Además, los niveles de LDH se elevaron significativamente 1,8, 2,9 y 3,2 veces en comparación con el grupo de control (Pang et al., 2022).

1.5.2.7. Zearalenona (ZEA) y sus metabolitos

Refiriéndose a los efectos de ZEA en las células SH-SY5Y, el ensayo MTT indicó que a las 6 h de incubación, ZEA no causó ningún daño celular. Sin embargo, redujo significativamente la viabilidad celular cuando la concentración superó los 20 µM a las 24 y 48 h, lo que resultó en una reducción de más del 50% en comparación con las células control. Se determinó que la concentración inhibitoria (IC_{50}) de ZEA fue de 17,4 µM (Pérez-Fuentes et al., 2021). Sin embargo, después de 24 h de tratamiento con ZEA, en un estudio previo se obtuvieron resultados similares en células SHSY5Y con una disminución significativa de la viabilidad celular incluso a una concentración más baja (25 µM) (Venkataramana et al., 2014). La viabilidad de las células SH-SY5Y disminuyó hasta en un 86% a 200 µM de ZEA (Venkataramana et al., 2014). En cuanto a la viabilidad celular en metabolitos de ZEA (α-ZEL y β-ZEL), se obtuvieron los siguientes valores de IC_{50} para α-ZEL: 20,8 a las 48 h, 14,0 a las 72 h y para β-ZEL: 94,3 a las 24 h, 9,1 a las 48 h y 7,5 a las 72 h (Agahi et al., 2020). En resumen, podemos afirmar que AA produce citotoxicidad en las células SH-SY5Y, a altas concentraciones

(5 mM o superiores), y que a concentraciones más bajas reduce la viabilidad celular de forma dependiente del tiempo y la dosis (Tabla 1). Por otro lado, las micotoxinas producen citotoxicidad a bajas concentraciones de 200 μM o menos, y esto depende del tiempo y la dosis en algunos casos. El compuesto más citotóxico fue el ENN B, seguido del DON y el ENN A. (Tabla 2). Por lo tanto, el AA y las micotoxinas afectan directamente a la viabilidad celular en todos los modelos celulares estudiados; las células más sensibles expuestas a AA fueron PC12, seguidas de SH-SY5Y y U-1240 MG.

Tabla 1. *Citotoxicidad en células SH-SY5Y. Condiciones de exposición, ensayos y efectos de*

Dosis	**Tiempo de exposición**	**Ensayos**	**Efectos**	**Referencias**
De 1 a 10 mM	8h	reactivo de recuento de células SF	10 mM de AA causa daño celular definitivo y muerte celular.	Komoike y Matsuoka 2016
De 1 a 5 mM	16h, 20h	Actividad Caspasa-3	La viabilidad de las células disminuyó de forma dosis-dependiente en todo el rango de la dosis (1-5 mM).	Sumizawa e Ingisu 2007.
2,5, 5, 7,5 y 10 mM	24h	CCK8	Reducción del 14%, 35%, 48% y 61%, respectivamente.	Yan et al., 2022
0,8, 20 y 500 µg/ml	24h	Subasta multimesa	Reducción del 60% y del 70% a las concentraciones más altas.	Ban et al., 2021
2, 2,5, 3, 4 y 5 mM	24h	Subasta multimesa	Reducción del 10%, 18%, 20%, 25% y 30%.	Yan et al., 2018
1,25, 2,5, 5 mM	24h	Subasta multimesa	Pérdida significativa de viabilidad celular.	Song et al., 2017
0 – 500 mM	24 h	Ensayo de viabilidad de células CellTiter-Blue	IC50 5 mM NI20 0.26 mM	Frimat et al., 2010
1, 10 y 100 µM	24 h	MTT, ATP, Caspasa	Reducción de la viabilidad celular de 100 µM.	Hallier-Vanuxeem et al., 2009
De 0,01 mM a 12 mM.	24h	Yoduro de propidio y calceína-AM	0,5–2,0 mM AA no mostró citotoxicidad. (4 mM) causó la pérdida significativa de neuronas HuD positivas (58 ± 7% del control) y el número de axones disminuyó a solo 21 ± 5% del control	Lourenssen et al., 2009
0,5–5 mM	24 h	Azul de tripano	Citotóxico a 5mM	Okuno et al., 2006
0,1-10 mM	24h	Ensayos de LDH	El AA no fue en gran medida citotóxico, excepto en las dosis más altas.	Hartley et al., 1997
0,1–1000 µM	48h	Ensayo de calceína-AM, βIII-tubulina y LDH	Reducción del 10% a 1 mM.	Popova et al., 2017
0 – 1000µM	72h	Contenido total de proteínas	>1000 µM IC50	Nordin-Andersson et al., 2003
0, 0,5, 1, 2, 5 y 10 mM	0, 6, 12, 24, 48 y 72 h	Azul de tripán y LDH	10 mM redujeron un 38% la viabilidad celular a las 6 h.	Chen et al., 2009
0, 0,1, 0,5, 1 y 2 mM	24,48,72h	Subasta multimesa	Reducción del 20%, 30% y 50%.	Chen y Chou 2015
1 - 1000 µM.	24, 48 y 72 h. 3, 6 y 10 días.	BradUrd, Sub-G_1, 3H-timidina y MTT	Reducción del 30% (48h) y del 90% (72h). Disminuya durante 3 y 6 días de exposición a una concentración de 100 µM o superior.	Attoff et al., 2016

AA: Acrylamide, **ATP:** adenosine triphosphate, **CCK8:** Cell counting kit-8, **IC_{50}:** 50%inhibition concentration, **LDH:** lactate dehydrogenase, **MTT:** (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), **NI_{20}:** 20% reduction in network formation.

Tabla 2. *Citotoxicidad en células SH-SY5Y. Micotoxinas, condiciones de exposición, ensayos y efectos*

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
DON	0 – 10 µM	6 h y 24 h	Subasta multimesa LDH	IC_{50}: 2,25 µM a las 24 horas; 1.5 a las 48h. No se observaron efectos citotóxicos después del tratamiento con DON	Pérez-Fuentes N., et al 2021
	0 – 200 µM	6 h y 24 h	Subasta multimesa LDH	IC_{50}: 120 µM a las 24 h; (El DON afectó la viabilidad de la célula en el modo de dosis y dependiente del tiempo). El nivel de LDH extracelular depende de las células muertas y es directamente proporcional.	Kumar Kalagatur N. et al., 2021
BEA	0,009–25 µM	24h 48 h y 72 h	Ensayo MTT	BEA IC_{50}: 2,5 a las 72 h	Agahi F et al., 2020a
	0,08 - 2,5 µM	24 h y 48 h	Ensayo MTT	La BEA disminuye un 43% de la viabilidad celular a 2,5 µM después de 48 h.	Agahi F et al., 2022
	0,08 - 2,5 µM	24 h y 48 h	Ensayo MTT	El valor de IC_{50} se alcanzó a las 72 h para 2,5 µM.	Juan C et al., 2020
	0,08 - 25 µM	24 h y 48 h	Ensayo MTT	IC_{50}: a las 24 h el valor de BEA IC_{50} fue de 3,2 µM, mientras que a las 48 h fue de 5 µM.	Montesano D et al., 2020
	0,1 - 30 µM	6 h 24 h y 48 h	Ensayo MTT Ensayo de LDH	IC_{50}: 1,9 µM durante 6 h, 1,7 µM durante 24 h y 1,5 µM durante 48 h (BEA disminuyó la viabilidad celular en más de un 50% a concentraciones superiores a 1,75 µM en todos los tiempos de incubación) Induciendo fugas de LDH a concentraciones superiores a 1 µM.	Pérez-Fuentes N et al., 2021
	0 – 2,5 µM	24 h 48 h y 72 h	Ensayo MTT	La IC_{50} a las 72 h fue de 2,5 µM.	Juan-García A et al 2021
ZEA α-ZEL y β-ZEL	0,39–100 µM	24h 48 h y 72 h	Ensayo MTT	IC_{50}: α-ZEL: 20,8 a las 48 h, 14,0 a las 72 h β-ZEL: 94,3 a las 24 h, 9,1 a las 48 h y 7,5 a las 72 h	Agahi F et al., 2020a
	0,4 - 12,5 µM	24 h y 48 h	Ensayo MTT	β-ZEL: se observó una disminución dependiente de la concentración en la viabilidad después de 48 h de exposición, en las concentraciones más altas de 6,25, 12,5 y 25 µM de 31% a 82%.	Agahi F et al., 2022
	0,1 - 30 µM	6 h 24 h y 48 h	Ensayo MTT Ensayo de LDH	ZEA presentó un IC_{50} de 17,4 µM	Pérez-Fuentes N et al., 2021

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
				En el caso de ZEA, se encontró un ligero aumento en la fuga de LDH a las 20 y 30 µM (9,1 ± _8,9% y 19,5 ± _4,3%, respectivamente) después de 24 h.	
	25, 50, 75, 100 y 200 µM		Ensayo MTT LDH	24 h de ZEN de 25 µM disminuyen la viabilidad celular. Disminuyó un 86% a 200 µM.	Venkataramana, S. et al., 2014
FB1	0,1 - 30 µM	6 h 24 h y 48 h	Ensayo MTT Ensayo LDH	FB1 redujo la viabilidad celular a la concentración más alta después de 48 h. El experimento de LDH se realizó a las 6 y 24 h, no se observaron efectos citotóxicos después del tratamiento con el FB1	Pérez-Fuentes N et al., 2021
	50 µM	12 h, 24 h y 48 h	Ensayo de LDH	FB1 llevó a la liberación de LDH a las 12 h.	Souren P et al., 2021
ENN	0,1 - 30 µM	6 h 24 h y 48 h	Ensayo MTT Ensayo LDH	ENN A IC_{50} de 2,4 µM a las 6h y 2,25 µM a las 24h. ENN B IC_{50} de 0,43 µM a las 24h. ENN Un aumento significativo de la liberación de LDH de las células SH-SY5Y a las 5 y 10 µM a las 6 h y a las 24 h. ENN B mostró efectos significativos sobre la liberación de LDH después de 24 h de tratamiento, produciendo un aumento de alrededor del 20% a concentraciones superiores a 0,25 µM	Pérez-Fuentes N et al., 2021
OTA	1, 10 o 100 µM	30, 60 min, 24 h,	LDH	Las actividades de LDH de las células SH-SY5Y tratadas con OTA 1, 10 o 100 µM fueron 128 ± 1, 125 ± 2 y 200 ± 1%.	Yoon S et al., 2009
	0,2 - 50 µM	24 h y 48 h	Ensayo MTT	Los valores de IC_{50} para 24 h y 48 h fueron 5,8 µM y 9,1 µM, respectivamente.	Penalva-Olcina R et al., 2022
	130–0,20 nM	24 h, 48 h y 72 h 7 días	Ensayo MTT	No se alcanzó el IC_{50}.	Frangiamone et al 2022
T-2	5 - 20 ng/mL T-2	1 – 48 h	Ensayo CCK-8 Ensayo de LDH	Se redujo significativamente a 81,9 %, 40,8 % y 35,5 % respectivamente para las células expuestas a 5, 10 y 20 ng/ml de toxina T-2 Los niveles de LDH aumentaron significativamente en 1.8, 2.9 y 3.2 veces en comparación con las celdas en el control del vehículo.	Pang et al., 2022

BEA: Beauvericin, **CCK8:** Cell counting kit-8, **DON:** Deoxynivalenol, **ENN'** s: enniatin, **FB1:** Fumonisin B1, **IC_{50}:** 50%inhibition concentration, **LDH:** lactate dehydrogenase, **MTT:** (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), **OTA:** Ochratoxin A, **ZEA:** Zearalenone, **α-ZEL:** alpha-zearalenol, **β-ZEL:** beta-zearalenol

1.6. Apoptosis

1.6.1. Acrilamida (AA)

Con el fin de evaluar la apoptosis celular, se monitorizaron los niveles de mononucleosomas y oligonucleosomas en células SH-SY5Y y U-1240 MG tratadas con AA. Esto reveló un aumento en el factor de enriquecimiento de fragmentos mononucleosomales y oligonucleosomales en ambas líneas celulares cuando se expusieron a concentraciones que oscilaron entre 0,5 y 10 mM de AA durante un período de 0 a 72 h. En las células SH-SY5Y, los niveles de fragmentos de ADN mostraron un aumento significativo con tiempos de exposición más largos y concentraciones más altas de AA. Por otro lado, las células U-1240 MG mostraron respuestas apoptóticas solo después de 48 h de exposición a todas las concentraciones de AA, excepto a 0,5 mM (Chen et al., 2009). Además, cuando las células SH-SY5Y se trataron con 2,5 mM de AA durante 24 h, hubo un aumento de aproximadamente cuatro veces en la tasa apoptótica total en comparación con el grupo de control (Bridgeman et al., 2023). Sin embargo, la inhibición de la c-Jun N-terminal quinasa (JNK) mediante un α inhibidor del factor nuclear B (NF-Bα) condujo a una reducción de la apoptosis temprana y tardía en las células tratadas con AA (Yan et al., 2019).

Para investigar el papel potencial y el mecanismo subyacente de la esfingosina quinasa 1 (SphK1) en la lesión nerviosa inducida por AA en células SH-SY5Y, se examinó la expresión de SphK1. Se observó que los niveles de SphK1 disminuyeron en correlación con el aumento de las concentraciones de AA. En concreto, tras la exposición a AA, las tasas de apoptosis y activación de SphK1 aumentaron proporcionalmente con la concentración de AA (Yu et al., 2022). A concentraciones de 1,25 mM y 2,5 mM, las tasas de apoptosis fueron de 3,06% ± 0,13% y 6,86% ± 0,67, respectivamente, mientras que en el grupo de activadores de SphK1, las tasas a las mismas concentraciones fueron de 2,12% ± 0,33% y 3,53% ± 0,17, respectivamente (Yu et al., 2022). Además, en un estudio relacionado, Ning et al. (2021), llevaron la investigación un paso más allá al utilizar AA como modelo para inducir la apoptosis en peces cebra, exponiéndolos a 10

mM de AA. Esto dio lugar a la observación de la neuroapoptosis inducida por AA a través de ensayos fluorescentes en el pez cebra (Ning et al., 2021).

1.6.2. Micotoxinas

En la siguiente sección se abordará la capacidad de alteracion del ciclo celular, concretamente de la generación de apoptosis de varias micotoxinas relevantes en seguridad alimentaria. Se analizarán específicamente los efectos de BEA, DON, ENN, FB1 y ZEA.

1.6.2.1. Beauvericina (BEA)

En cuanto a las alteraciones del ciclo celular, la BEA redujo significativamente el porcentaje de células viables, produciendo un aumento de la muerte celular apoptótica del 55,9% ± del 8,6% (Pérez-Fuentes et al., 2021). Resultados similares fueron obtenidos por Agahi et al. (2021), quienes afirmaron que las células apoptóticas/necróticas aumentaron para ambos tiempos de exposición, que fue de hasta un 89% para 24 h y hasta un 38,8% para 48 h. Además, Agahi et al. (2021) observaron que después de 48 h de exposición, hubo un aumento notable de las células necróticas a 0,39 y 0,78 µM casi dos veces en comparación con las células de control.

1.6.2.2. Deoxinivalenol (DON)

En el caso del DON, Kalagatur et al. (2021) informaron que una dosis de 120 µM indujo daño en el ADN y condujo a la formación de núcleos apoptóticos.

1.6.2.3. Enniatina A and Enniatina B (ENN A and ENN B)

ENN A y ENN B redujeron la viabilidad celular, produciendo un aumento de la muerte celular apoptótica de 49,2 ± 8,2% y 46,0 ± 9,3%, respectivamente (Kalagatur et al., 2021).

1.6.2.4. Zearalenona (ZEA) y sus metabolitos

ZEA, por otro lado, no produjo un aumento significativo en la muerte celular a las concentraciones ensayadas (Agahi et al., 2021b). Sin embargo, las células tratadas con α-ZEL aumentaron significativamente en las células apoptóticas y apoptóticas/necróticas a las 24 h. Y β-ZEL mostró un aumento significativo en las células apoptóticas a la concentración más alta ensayada (12,5 μM), tanto a las 24 como a las 48 horas (Agahi et al., 2021). En el caso de la AA, se ha demostrado que incrementa la tasa de apoptosis en células neuronales, como SH-SY5Y y U-1240 MG, de forma proporcional a la concentración y al tiempo de exposición. La activación de la enzima esfingosina quinasa 1 (SphK1) ha mostrado efectos protectores, aumentando la supervivencia celular y reduciendo la apoptosis en células SH-SY5Y. Además, estudios in vivo han evidenciado que la AA puede inducir neuroapoptosis en el modelo de pez cebra (Tabla 3). Las micotoxinas como BEA, ENNs y metabolitos ZEA (α-ZEL y β-ZEL) también han demostrado reducir la viabilidad celular en células SH-SY5Y y aumentar la muerte celular por apoptosis (Tabla 4).

Tabla 3. *Apoptosis en células SH-SY5Y. Condiciones de exposición, ensayos y efectos de AA.*

Dosis	Hora	Ensayos	Efectos	Referencias
2,5 mM	24h	Anexina V-FITC/PI apoptosis	En el grupo incubado con AA, la tasa de apoptótica temprana fue del 5,5%, la tasa de apoptótica tardía fue del 11,8% y la tasa de apoptótica total aumentó aproximadamente 4 veces más que en el grupo de control.	Yan et al., 2022
2.5mM	24h	Anexina V-FITC/PI apoptosis	Los efectos reguladores de JNK, p38 y NF-κB sobre AA indujeron la apoptosis de las células SH-SY5Y.	Yan et al., 2018
0,5–10 mM	De 0 a 72 h	Detección de fragmentación de ADN (mononucleosomas y oilgonucleosomas)	El nivel de fragmentos de ADN aumentó significativamente con el aumento de las concentraciones de AA y el tiempo de exposición.	Chen et al., 2009

Dosis	Hora	Ensayos	Efectos	Referencias
1,25-2,5mM	24h	Anexina V-FITC/PI apoptosis	Aumenta con la disminución de las concentraciones de AA.	Yu et al., 2022
10 mM	24h	ELISA y citometría	El AA induce neuroapoptosis en peces cebra y produce radicales libres.	Ning et al., 2021

AA: Acrylamide, **DNA:** Deoxribonucleic Acid, **JNK:** c-Jun N-terminal kinase, **NF-κB:** nuclear factor κB, **SH-SY5Y:** neuroblastoma cell line.

Tabla 4. Apoptosis en células SH-SY5Y. Condiciones de exposición, ensayos y efectos a micotoxinas.

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
DON	0 – 10 µM	6 h y 24 h	Ciclo celular, FC	El DON no produjo un aumento significativo en la muerte celular a las concentraciones ensayadas.	Pérez-Fuentes N., et al 2021
	0 – 200 µM	6 h y 24 h	Apoptosis por FC	Formación de núcleos apoptóticos inducidos por DON.	Kumar Kalagatur N. et al., 2021
BEA	2,5, 1,25, 0,78 y 0,39 µM	24 h y 48 h	Ciclo celular Apoptosis/necrosis	Detención del ciclo celular en G0/G1. Las células apoptóticas/necróticas aumentaron hasta un 89 % durante 24 h y hasta un 38,8 % durante 48 h. Se observó una promoción de la necrosis en las concentraciones más bajas.	Agahi F et al., 2021b
	0,1 - 30 µM	6 h 24 h y 48 h	Análisis de FC (apoptosis)	BEA redujo significativamente el porcentaje de células viables, produciendo un aumento de la muerte celular apoptótica del 55,9 ± del 8,6%.	Pérez-Fuentes N et al., 2021
ZEA α-ZEL y β-ZEL	12,5, 6,25, 3,12 y 1,56 µM	24 h y 48 h	Ciclo celular Apoptosis/necrosis	Aumento dependiente de la concentración en la fase G0/G1 a las 24 h. α-ZEL aumentó las células apoptóticas y apoptóticas/necróticas a las 24 h. β-ZEL aumenta las células apoptóticas expuestas a 12,5 µM después de 24 y 48 h.	Agahi F et al., 2021b
	0,1 - 30 µM	6 h 24 h y 48 h	Análisis de FC (apoptosis)	El ZEA no produjo un aumento significativo de la muerte celular a las concentraciones ensayadas.	Pérez-Fuentes N et al., 2021
FB1	0,1 -100 µM	48 h, 72 h y 144 h	Viabilidad celular (propidio) Yoduro)	Disminución de la viabilidad de las células SH-SY5Y después de la incubación con 100 µM de FB1 durante 48 a 144 h.	Stockmann-Juvala H et al 2004
	0,1 - 30 µM	6 h 24 h y 48 h	Análisis de FC (apoptosis)	FB1 no produjo un aumento significativo en la muerte celular a las concentraciones probadas	Pérez-Fuentes N et al., 2021
	50 µM	12 h, 24 h y 48 h	Ensayo de apoptosis (FC)	Apoptosis inducida por FB1 en células SHSY-5Y	Souren P et al., 2021

BEA: Beauvericin, **DON:** Deoxynivalenol, **ENN'** s: enniatin, **FB1:** Fumonisin B1, **FC:** Flow cytometry, **OTA:** Ochratoxin A, **ZEA:** Zearalenone, **α-ZEL:** alpha-zearalenol, **β-ZEL:** beta-zearalenol.4. Oxidative Stress

1.7. Estrés oxidativo

1.7.1. Acrilamida (AA)

El estudio de las ROS en la línea celular SH-SY5Y reveló una acumulación tras la exposición a AA (Komoike et al., 2016). Estos resultados están respaldados por los reportados que indican una disminución intracelular en la producción de glutatión (GSH) de manera dependiente de la dosis y un aumento en la generación de malondialdehído (MDA) y ROS cuando las células SH-SY5Y fueron tratadas con 2.5 y 5 mM de AA (Yan et al., 2019). Además, cuando las células SH-SY5Y se expusieron a AA a 2,5 mM durante 24 h, causaron estrés oxidativo, como lo revela el claro aumento de las ROS celulares y el nivel de MDA y una disminución significativa del contenido de GSH (Yan et al., 2022).

1.7.2. Micotoxinas

En la siguiente sección se abordará la capacidad de alteración del estrés oxidativo, de varias micotoxinas relevantes en seguridad alimentaria. Se analizarán específicamente los efectos de BEA, DON, ENN, FB1, OTA, T-2 y ZEA.

1.7.2.1. Beauvericina (BEA)

Ante los efectos sobre la citotoxicidad y apoptosis de las micotoxinas, el estrés oxidativo fue analizado por varios autores. Agahi et al. (2020) estudiaron la producción de ROS y la relación GSH/GSSG en la que BEA produjo una ligera disminución a 1,25 y 2,5 µM, de 45 a 120 min en comparación con el control, y la relación GSH/GSSG aumentó después de 24 h en células expuestas a BEA de 103% a 142%. Posteriormente, en la misma línea de investigación, estudiaron la actividad de GPx, GST, CAT y SOD (Agahi et al., 2021). Los resultados mostraron un aumento que varió de 9 a 17 veces y de 2 a 9 veces después de 24 h y 48 h, respectivamente. Además, la BEA aumentó la actividad de GST a dosis superiores a 0,78 µM durante 48 h de exposición en un 4-32%. La actividad del CAT no se alteró cuando las células se expusieron a la micotoxina BEA. Finalmente, la actividad de SOD aumentó significativamente después de estar

expuesto a BEA después de 48 h hasta 1 veces. En otro estudio (Pérez-Fuentes et al., 2021), se analizó la medición del potencial de membrana mitocondrial, concluyendo que el BEA tenía la capacidad de despolarizar la membrana mitocondrial a concentraciones que oscilaban entre 2,5 a 10 µM a las 6 h y 24 h.

1.7.2.2. Deoxinivalenol (DON)

Además, Kalagatur et al. (2021) informaron que a 120 µM, el DON indujo ROS y estrés oxidativo sobre la inducción de LPO y el agotamiento de las enzimas antioxidantes (GSH, CAT y SOD). Además, el DON indujo la pérdida de MMP (Kalagatur et al., 2021).

1.7.2.3. Enniatina B (ENN B)

A las 24 h, esta toxina emergente afectó el valor de DYm en todas las concentraciones analizadas. El tratamiento con ENN B alteró la DYm a las dosis más altas empleadas (5 y 10 µM) a las 6 h de incubación y provocó diferencias a las 24 h (Pérez-Fuentes et al., 2021).

1.7.2.4. Fumonisina B1 (FB1)

En términos de estrés oxidativo, el tratamiento con FB1 resultó en una producción notablemente mayor de ROS en comparación con las células de control. Además, midieron la cantidad de acumulación de ROS en las mitocondrias y descubrieron que la terapia con FB1, independientemente de la duración del tratamiento, aumentaba la acumulación de ROS en las mitocondrias (Paul et al., 2021). Es interesante notar que la adición de 0,5 µM de FB1 a las células de neuroblastoma aumentó la tasa de ROS en 1,2 veces. En las células de neuroblastoma, el aumento de la concentración de FB1 a 5 y 50 µM no activó la tasa de fluorescencia de HEt de forma dependiente de la dosis (Domijan et al., 2011). Por otro lado, en todos los puntos temporales (24, 48, 72, 96, 120 y 144 h) y concentraciones de fumonisina (0,1 a 100 µM) empleados, el tratamiento con FB1 no tuvo ningún efecto sobre la formación de ROS (Stockmann-Juvala et al., 2009).

Cuando los niveles de radicales libres son altos, las ROS pueden ser dañinas para las células porque reducen significativamente la cantidad de antioxidantes endógenos. Una de las principales vías antioxidantes del SNC es suministrada por GSH. El estrés oxidativo hace que el GSH se oxide, y la enfermedad del SNC es el resultado de la deficiencia de GSH (Penalva-Olcina et al., 2022). Se observaron niveles reducidos de GSH (61% de los controles) en las células SH-SY5Y después de un período de incubación de 144 h con 100 µM de FB1, pero no en momentos anteriores ni con dosis más bajas de FB1 (Agahi et al., 2022).

Después de incubar con 100 µM FB1 durante 24 h, las células SH-SY5Y ya mostraron concentraciones aumentadas de MDA (Pérez-Fuentes et al., 2021). Después de un período de incubación de 72 h, las células expuestas a 10 µM de FB1 mostraron niveles elevados de MDA. Los niveles de MDA en los otros periodos de tiempo no difirieron estadísticamente de los de los controles (Pérez-Fuentes et al., 2021). En un estudio diferente, el nivel de Ca2+ celular de las células de neuroblastoma aumentó considerablemente después de la exposición a FB1 en comparación con el grupo de control. Tras el estrés del RE, se libera Ca2+ celular, lo que provoca la pérdida del potencial de la membrana mitocondrial y, en las células de neuroblastoma, provoca la muerte celular (Paul et al., 2021). Sin embargo, Pérez-Fuentes et al. (2021), encontraron que el tratamiento con 30 µM de FB1 no resultó en alteraciones apreciables en el DYm de las células SH-SY5Y durante ninguno de los dos periodos de incubación. Si bien la fluorescencia de MitoSOX aumentó en todas las dosis, solo la concentración más baja (0,5 µM) tuvo un impacto perceptible (Domijan et al., 2011).

1.7.2.5. Ocratoxina A (OTA)

En relación con la producción de ROS, Yoon et al. (2009) demostraron que el tratamiento con 100 µM de OTA durante 30 y 60 minutos, seguido de la carga celular con 2 mM de DCF-DA durante 30 minutos, resultó en un aumento de la intensidad de fluorescencia de DCF-DA en comparación con las células control no tratadas. Estos hallazgos sugieren que las células SH-SY5Y expuestas a OTA estuvieron sometidas a condiciones de estrés oxidativo.

1.7.2.6. T-2 Toxina

En esta investigación, los niveles de ROS aumentaron drásticamente a 3,8 y 5,0 veces, respectivamente, en las células tratadas con 5 y 10 ng/mL de la toxina T-2. Como resultado, para las células tratadas con 5 y 10 ng/mL de la toxina T-2, la proporción de GSH a GSSG se redujo drásticamente a 79,8% y 60,7%, respectivamente. El contenido de ATP disminuyó a 66,7% y 51,5% a las concentraciones de 5 y 10 ng/mL, respectivamente, mientras que el potencial de membrana mitocondrial disminuyó a 60,7% y 41,5% a las mismas concentraciones (Pang et al., 2022).

1.7.2.7. Zearalenona (ZEA) y sus metabolitos

La administración de ZEN aumentó drásticamente la formación de ROS en las células SH-SY5Y en relación con el estrés oxidativo (*Venkataramana et al., 2014*). Además, la administración de ZEN condujo a un aumento dependiente de la dosis en la pérdida de MMP y la peroxidación lipídica (*Agahi et al., 2022; Pérez-Fuentes et al., 2021).* En cuanto a los metabolitos de ZEA, el α-ZEL a 25 μM elevó las ROS de 5 a 60 min y disminuyó moderadamente de 90 a 120 min en comparación con su control. Por el contrario, el -ZEL disminuyó en 12,5 μM durante un período de 90 minutos (*Agahi et al., 2020*). Después de 24 h, la relación GSH/GSSG en las células expuestas a micotoxinas en medios frescos aumentó drásticamente del 111% al 148%, y para α-ZEL y β-ZEL, del 68% al 131%, respectivamente (*Agahi et al., 2020*). Profundizando en nuestra comprensión de las consecuencias nocivas de los metabolitos de ZEA, el estudio realizado por Agahi et al. (2021) examinó los efectos de α-ZEL y β-ZEL en la actividad de las enzimas en las células SH-SY5Y. Los resultados mostraron que la exposición a α-ZEL y β-ZEL aumentó la actividad de GPx, CAT y GST en todas las concentraciones examinadas, y que SOD y β-ZEL aumentaron la actividad de SOD y GST después de 48 h.

En resumen, el AA produce un aumento de las ROS celulares y una reducción del GSH intracelular a concentraciones superiores a 2,5 mM durante más de 24 h (Tabla 5). Las micotoxinas (DON, FB1, OTA, toxina T-2 y ZEA) produjeron un aumento de las ROS

celulares y de las ROS mitocondriales en el caso de FB1, y una reducción de la relación GSH a concentraciones de 120 µM para DON, 0,5 µM para FB1, 100 µM para OTA, 5 y 10 ng/mL para la toxina T-2 y 12,5 µM para α-ZEL y β-ZEL (Tabla 6).

Tabla 5. *Estrés oxidativo. Dosis de AA, tiempo de exposición, ensayos, efectos y referencias.*

Dosis	Hora	Ensayos	Efectos	Referencias
10 mM	6h	ROS	La exposición induce la acumulación de ROS en las células SH-SY5Y	Komoike y Matsuoka 2016
2,5 mM y 5 mM	24 h	GSH, MDA y ROS	2,5 y 5 mM disminuyeron la producción intracelular de GSH y aumentaron la generación de MDA y ROS.	Yan et al., 2018
2,5 mM	24 h	ROS (DCFH-DA) y LPO (MDA, GSH)	Producción máxima de ROS; 2,5 mM (+ 40%). Contenido de MDA: +100% 2,5 Contenido de GSH: -50% 2,5 mM.	Yan et al., 2022

DCFH-DA: Dichloro-dihydro-fluorescein diacetate, **GSH:** glutatión, **LPO:** lipid peroxidation, **MDA:** malondialdehyde, **ROS:** reactive oxygen species.

Tabla 6. Estrés oxidativo. Dosis de micotoxinas, tiempo de exposición, ensayos, efectos y referencias.

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
DON	0 – 200 µM	6 h y 24 h	Generación de ROS por fluorometría Niveles de LPO y enzimas antioxidantes MMP	Efecto dosis-dependiente del DON sobre la generación de ROS intracelulares durante 6 y 24 h. El DON induce estrés oxidativo sobre la inducción de LPO y el agotamiento de las enzimas antioxidantes. Pérdida de MMP inducida por DON	Kumar Kalagatur N. et al., 2021
BEA	2,5, 1,25, 0,78 y 0,39 µM	2 h, 24 h y 48 h	ROS GSH	Para BEA, las ROS disminuyen a 1,25 y 2,5 µM, de 45 a 120 min respecto al control. La relación GSH/GSSG aumentó después de 24 h en células expuestas a BEA de 103 % a 142 %.	Agahi F et al., 2020b
	2,5, 1,25, 0,78 y 0,39 µM	24 h y 48 h	Actividad de la glutatión peroxidasa (GPx) Actividad del glutatión S-transferasa (GST) Actividad de la catalasa (CAT) Actividad de la superóxido dismutasa (SOD)	Alteración de la actividad de las enzimas antioxidantes. Para la actividad de GPx, BEA promovió un aumento de 9 a 17 veces después de 24 h y de 2 a 9 veces durante 48 h. En relación con la actividad de GST, se obtuvo un ligero aumento (4-32% a las 48h) mientras que para SOD, se mostró un aumento de 2,5 veces a las 48h. No se observaron diferencias significativas para el TAC.	Agahi F et al., 2021a
	0,1 - 30 µM	6 h 24 h y 48 h	MMP	Alteración de la membrana mitocondrial a 2,5-10 µM durante 6-24 h.	Pérez-Fuentes N et al., 2021
ZEA α-ZEL y β-ZEL	25, 12.5, 6,25 y 3,12 µM	120 minutos 24 h y 48 h	ROS GSH	α-ZEL: a 25 µM aumento de 5 a 60 min y de 90 a 120 min de disminución. β-ZEL: disminución de 5 a 90 min en 12,5 µM y a 25 µM después de 15 y 30 min y disminución de 60 a 120 min. La relación GSH/GSSG aumentó después de 24 h en todas las concentraciones, del 111 % al 148 %, y del 68 % al 131 % para el α-ZEL y el β-ZEL, respectivamente.	Agahi F et al., 2020b
	12,5, 6,25, 3,12 y 1,56 µM	24 h y 48 h	Actividad de la glutatión peroxidasa (GPx) Actividad del glutatión S-transferasa (GST) Actividad de la catalasa (CAT) Actividad de la superóxido dismutasa (SOD)	α-ZEL y β-ZEL: la actividad de GPx aumentó de 13,5 a 23 veces para α-ZEL y de 9 a 17 veces para β-ZEL. Para 24 h y 48 h El GST aumentó después de 48 h de exposición al β-ZEL a 3,12 y 12,5 µM en un 22% y un 102%, respectivamente. La actividad de CAT aumentó a α-ZEL y β-ZEL después de 48 h de exposición (de 0,4 a 1,4 veces para α-ZEL y de 1 a 4,2 veces para β-ZEL) en todas las concentraciones analizadas. La actividad de SOD aumentó después de 48 h de exposición en todas las concentraciones analizadas. En consecuencia, para α-ZEL hasta 1,4 veces, y para β-ZEL hasta 2,5 veces.	Agahi F et al., 2021a

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
	0,1 - 30 µM	6 h 24 h y 48 h	MMP	ZEA produjo una ligera disminución a las 30 µM después de 24 h de incubación.	Pérez-Fuentes N et al., 2021
	25, 50, 75, 100 y 200 µM		ROS Ensayo de peroxidación lipídica MMP	ZEN aumentó la generación de ROS. El ZEN aumentó la peroxidación lipídica de forma dependiente de la dosis. ZEN aumentó la pérdida de MMP de manera dependiente de la dosis.	M Venkataramana a, S. et al., 2014
FB1	0,1 -100 µM	48 h, 72 h y 144 h	ROS Peroxidación lipídica GSH	FB1 no afectó a la producción de ROS. Se observó un aumento de las concentraciones de MDA en las células SH-SY5Y ya después de una incubación de 24 h con 100 µM de FB1. Después de una incubación de 72 h, se observó un aumento de los niveles de MDA en las células tratadas con 10 µM de FB1. Se observaron niveles disminuidos de GSH (61% de los controles) a 100 mM FB1 durante 144 h.	Stockmann-Juvala H et al 2004
	De 0,5 a 200 µM	24 h y 48 h	ROS MitoSOX	0,5, 5 y 50 M, aumentaron significativamente la producción de ROS. Todas las dosis aumentaron la fluorescencia de MitoSOX, solo la concentración más baja (0,5 µM) tuvo un efecto significativo.	Domijan A-M et al., 2011
	0,1 - 30 µM	6 h 24 h y 48 h	MMP	FB1 no generó cambios significativos en ΔΨm de las células SH-SY5Y en ambos momentos de incubación.	Pérez-Fuentes N et al., 2021
	50 µM	12 h, 24 h y 48 h	ROS Nivel de superóxido mitocondrial Medición de Ca2+	Aumento de la producción de ROS después del tratamiento con FB1. El tratamiento con FB1 aumentó la acumulación de ROS mitocondrial, independientemente del tiempo de tratamiento. La generación de ROS inducida por FB1 se anuló fuertemente cuando se trató un eliminador de ROS, N-acetil-L-cisteína (NAC), durante 24 h (Halasi et al., 2013). También comprobamos el nivel de acumulación de ROS mitocondrial, que es un marcador bien conocido del estado redox celular (Paul et al., 2015). El nivel de Ca2+ celular aumentó después de la exposición a FB1.	Souren P et al., 2021

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
ENN A	0,1 - 30 µM	6 h 24 h y 48 h	MMP	ENN A produjo diferencias significativas en comparación con las células control a 5 y 10 µM después de 6 h de incubación (79.0 ± _8.9% y 67.7 ± _14.5%, respectivamente. A las 24 h, ENN A afectó a ΔΨm en todas las concentraciones ensayadas. ENN B alteró ΔΨm a las dosis más altas empleadas (5 y 10 µM) después de 6 h de incubación y causó diferencias significativas con respecto al control en todas las concentraciones ensayadas a las 24 h	Pérez-Fuentes N et al., 2021
OTA	OTA de 1, 10 o 100 µM	30, 60 min, 24 h,	ROS	100 µM OTA durante 30 y 60 min. indicaba la evocación del estrés oxidativo.	Yoon S et al., 2009
	0,1, 0,25, 0,5, 1,0 y 2,5 µM	24 h, 48 h	MMP	Pérdida del potencial de membrana de las mitocondrias.	Zhang X et al 2009
T-2	5 - 20 ng/mL T-2	1 – 48 h	Nivel de ROS, Relación GSH/GSSH, Contenido ATP MMP	Los niveles de ROS con 5 y 10 ng/mL de toxina T-2 aumentaron significativamente a 3,8 y 5,0 veces, respectivamente. La relación entre GSH y GSSG disminuyó a 79,8% y 60,7% a 5 y 10 ng/mL, respectivamente. La toxina T-2 de 5 y 10 ng/mL disminuyó el potencial de membrana mitocondrial a 60,7% y 41,5%, mientras que el contenido de ATP disminuyó a 66,7% y 51,5%.	Pang et al., 2022

ATP: adenosine triphosphate, **BEA:** Beauvericin, **DON:** Deoxynivalenol, **ENN'** s: enniatin, **FB1:** Fumonisin B1, **GSH:** glutatión, **LPO:** lipid peroxidation, **MDA:** malondialdehyde, **MMP:** Mitochondrial membrane potential, **ROS:** reactive oxygen species, **OTA:** Ochratoxin A, **ZEA:** Zearalenone, **α-ZEL:** alpha-zearalenol, **β-ZEL:** beta-zearalenol

1.8. Degeneración de la red neuronal

Otra forma de evaluar cómo el AA ejerce efectos a nivel neuronal es explicando si degrada o impide el desarrollo de la red neuronal durante el proceso de diferenciación; un estudio se recoge que la degeneración no se notó durante las primeras 48 h después de recibir tratamientos con AA de 1 mM, pero que los niveles de la red habían disminuido considerablemente a las 72 h (Forsby 2011). En este caso, el deterioro de la red no pudo detenerse mediante el tratamiento conjunto con BDNF o calpeptina. Posteriormente se informaron resultados similares que demostraron que el número de neuritas se redujo significativamente después de 100 nM después de 3 días de exposición y después de 6 días de exposición a 10 pM de AA (Attoff et al., 2016), mostrando un deterioro en el crecimiento de las neuritas de una manera dependiente del tiempo y la dosis en estudios futuros (Ban et al., 2021). Además, 0,5 mM de AA causó un acortamiento en la morfología de las neuritas SH-SY5Y, mientras que 1 y 2 mM de AA dieron lugar a células sin extensiones morfológicas de neuritas (Chen y Chou 2015). Se encontraron resultados similares cuando las células se trataron con 0,25 mM de AA, causando una degeneración de neuritas del 50% a las 24 h (Nakagawa-Yagi et al., 2001). A las 96 h con 4 mM de AA, las neuritas SH-SY5Y se redujeron hasta en un 67,7% (Lourenssen et al., 2009). Por lo tanto, AA induce la degeneración de la red neuronal de una manera dependiente de la dosis y el tiempo (Nordin-Andersson et al., 1998).

Con el fin de obtener resultados más precisos, Nordin-Andersson et al. (1998), expusieron células diferenciadas de neuroblastoma humano (SH-SY5Y) a una serie de contaminantes, entre ellos AA, estudiando la citotoxicidad general (IC_{20}) y el efecto degenerativo de las neuritas (ND20), concluyendo a las 72 h que el IC_{20} era de 670 mM y el ND20 de 250 mM. Así, los valores de ND20 para AA fueron significativamente (65%) inferiores a los de IC_{20}, concluyendo la inducción de axonopatía. Más tarde, en 2003, durante el tratamiento con AA, se observaron citotoxicidad basal, modificaciones morfológicas y cambios en las actividades fisiológicas y neuroquímicas celulares en células de neuroblastoma humano diferenciado (SH-SY5Y) (Nordin-Andersson et al., 2003). Después de 72 h de exposición, AA causó una disminución del 20% en el número

de neuritas por célula a 0,21-0,25 mM y una disminución del 20% en la tasa de síntesis de proteínas a 0,17 mmol/L. Además, hubo un aumento del 49% y del 38% en los flujos basales de concentración de calcio intracelular ($[Ca^{2+}]i$), de 0,25 mmol/L y 0,5 mmol/L, respectivamente. Las células SH-SY5Y se recuperaron 48 h después de que se suspendiera la exposición a AA; es decir, su nivel basal de la concentración de calcio $[Ca^{2+}]$, la tasa de síntesis de proteínas y el número de neuritas por célula eran todos similares a los de las células de control. En conjunto, el AA altera el crecimiento de neuritas en la línea celular SH-SY5Y durante la diferenciación de una manera dependiente del tiempo y la concentración, especialmente después de 72 h de exposición. Sin embargo, a las 48 h después del cese de la exposición a AA, la célula SH-SY5Y se recupera (Tabla 7).

Tabla 7. Axonopatía en células SH-SY5Y. Condiciones de exposición,

Dosis	Hora	Ensayos	Efectos	Referencias
0,5 mM o 1 mM	0, 24, 48 y 72 h	Integridad de la red neuronal (conexiones de neuritas)	Con los tratamientos con AA 1 mM, la degeneración no fue evidente durante las primeras 48 h, pero a las 72 h los niveles de la red se redujeron significativamente. El tratamiento concomitante con calpeptina o BDNF fue insuficiente para prevenir la degeneración de la red.	Hardelauf et al., 2011
100 nm y 10 pm	3 y 6 días	Microscopio de contraste de fases	AA perjudica el crecimiento de neuritas en la línea celular SH-SY5Y durante la diferenciación de una manera dependiente del tiempo y la concentración.	Attoff et al., 2016
0,8, 20 y 500 µg/ml	24h	Microscopio de contraste de fases	El número y la duración de los procesos mostraron una tendencia gradualmente decreciente	Ban et al., 2021
0.5mM	24,48,72h	Indicadores bioquímicos de cambios morfológicos	Hizo que las células SH-SY5Y exhibieran una morfología de neurita más corta, mientras que 1 y 2 mM dieron como resultado células sin extensiones morfológicas de neurita	Chen y Chou 2015
0,1-1,0 mmol/L	72h	Cuantificación de neuritas	ND20 0,21 mmol/L	Nordin-Andersson et al., 2003
0.25mM	24h	Axonopatía (ensayo de degeneración de neuritas	0,25 mM 50% degeneración de neuritas.	Forsby 2011
4 mM	24h 96h	Número de neuronas y axones	El tratamiento previo con 4 mM de AA redujo aún más el número de neuronas en 96 h, hasta el 67,7% del control emparejado o una disminución adicional del 15% del valor a las 24 h	Lourenssen et al., 2009
100, 250 y 500 µm.	72 h	Axonopatía	La misma concentración y tiempo de exposición disminuyó el número de neuritas por célula en un 24%.	Forsby y Blaauboer 2007
6 mM	8 h	Axonopatía	Dependiente de la dosis de AA en concentraciones de 2,5 a 10 mM	Nakagawa-Yagi et al., 2001
250 µm 670 µm	72h	Axonopatía	AA indujo una disminución dependiente de la concentración en el número de neuritas / células. ND20: 250 IC20: 670	Nordin-Andersson et al., 1998
0,1-10 mM	4h, 24h, 48h	Ensayo de morfología	Las células diferenciadas fueron más sensibles a los efectos de AA (1mM) y 2,5-hexanedione en comparación con las células indiferenciadas.	Hartley et al., 1997

AA: Acrylamide, **BDNF:** brain derived neurotrophic factor, **IC_{20}:** concentration causing 20% protein reduction, **ND_{20}:** concentration causing 20% neurite degeneration.

1.9. Vías de señalización

Para comprender completamente cómo la AA interfiere a nivel neuronal, varios estudios examinaron la participación de diferentes factores de transcripción cruciales en el neurodesarrollo, la diferenciación celular, la apoptosis y las respuestas inflamatorias. Estos incluyeron la unión al elemento de respuesta de cAMP (CREB), las proteínas quinasas activadas por mitógenos (MAPKS), JNK, PERK-eIF2 y la proteína homóloga C/EBP (CHOP), junto con el factor nuclear NF-β. AA se estudió con la señalización de la proteína CREB durante la diferenciación neuronal, concluyendo que en la vía de señalización CREB la expresión de 17 genes cambió significativamente después de exposiciones a 1 o 70 μM, informando que el AA interfiere con importantes marcadores neuronales colinérgicos y dopaminérgicos durante la diferenciación, que se regularon a la baja después de la exposición a 70 μM de AA (Attoff et al., 2020).

En otros estudios (Yan et al., 2019), se evaluaron los niveles intracelulares de interleucina 6 (IL-6) y factor de necrosis tumoral alfa (TNF-α) en células SH-SY5Y con el fin de analizar los posibles efectos proinflamatorios de la AA. Los resultados mostraron que, en comparación con el valor de control, el contenido de TNF-α aumentó significativamente después del tratamiento con 2,5 y 5 mM de AA; sin embargo, el nivel de IL-6 solo aumentó significativamente en el grupo de 5 mM de AA en comparación con el grupo de control. Además, la NF-β nuclear y la vía de señalización de proteínas quinasas activadas por mitógenos (MAPK) con JNK y p38 fueron activadas por AA.

El mismo grupo de investigación estudió la inducción de AA en la agregación de la proteína TAU fosforilada, la reducción de la proteína CREB fosforilada y la regulación positiva de la relación *Bax/Bcl-2* en células SH-SY5Y a las mismas concentraciones informadas anteriormente. Además, AA indujo glucógeno sintasa quinasa-3 (GSK-3) y reguló al alza el factor de transcripción activador 4 (ATF4) y CHOP en células SH-SY5Y. También activó la proteína quinasa quinasa endoplásmica tipo ARN retículo quinasa (PERK)-Factor de iniciación eucariota 2 alfa (eIF2α) señalización (Yan et al., 2022). Sin embargo, se encontró que 1,25, 2,5 y 5 mM de AA aumentaban la escisión

de la caspasa-3 y la enzima poli ADP ribosa polimerasa (PARP) (las caspasas iniciadoras y efectoras en la vía apoptótica intrínseca, respectivamente). También disminuyeron la fosforilación de Akt en las células SH-SY5Y, lo que impidió la activación de la vía PI3K/Akt (Song et al., 2017). Los niveles de GSH se restauraron y la estimulación de H2O2 fue inhibida por el pretratamiento de LA, lo que contrarrestó las alteraciones inducidas por AA en la pérdida de GSH y la producción de H2O2. La regulación negativa de la fosforilación de la proteína quinasa activada por AMP (AMPK) y la fosforilación de la glucógeno sintasa quinasa-3 beta (GSK3) fue inducida por AA de manera dependiente de la dosis; AA provocó déficits de energía en las células SH-SY5Y mediante la regulación de la cascada AMPK/GSK3, lo que provocó una perturbación de Ca2+, agotamiento de ATP y deterioro de la señalización de CREB/BDNF. Además, la sirtuina 1 (Sirt1) y el coactivador 1 del receptor activado por el proliferado de peroxisomas (PGC-1) fueron regulados a la baja por el AA; sin embargo, el AA provocó autofagia celular en términos de la conversión de la cadena ligera de la proteína asociada a los microtúbulos 3 (LC3)-I a LC3-II y la expresión de la proteína beclin-1. Además, AA indujo la sobreexpresión de prostaglandina-endoperóxido sintasa 2 (COX-2) y óxido nítrico sintasa inducible (iNOS), y AA redujo la fosforilación de quinasas reguladas por señales extracelulares (ERK) mientras intensificaba la fosforilación de JNK y p38 en MAPKs. Además, AA medió una reducción en la translocación nuclear del factor 2 relacionado con el factor eritroide 2 (Nrf2) y en la expresión de la proteína 1 asociada a la ECH (Keap1) similar a Kelch en el citoplasma, lo que resultó en la regulación negativa de la expresión de las enzimas de fase II, a saber, HO-1 y NAD(P)H Quinona Deshidrogenasa 1 (NQO1) (Song et al., 2017).

Los estudios publicados constatan que la AA moduló varias vías de señalización relacionadas con la diferenciación, la inflamación y la apoptosis, perturbando factores transcriptómicos como la vía de señalización nuclear NF-B y MAPKs con activación de JNK y p38, la sobreexpresión de COX-2 e iNOS, y la regulación negativa de AMPK y GSK3. AA también inhibió la activación de la vía PI3K/Akt y reguló a la baja Sirt1 y

PGC-1. Además, interfirió con la vía de señalización CREB, un importante marcador neuronal colinérgico y dopaminérgico durante la diferenciación de SH-SY5Y (Tabla 8).

Tabla 8. *Vías de señalización en células SH-SY5Y. Condiciones de exposición, ensayos y efectos de AA.*

Dosis	Hora	Ensayos	Efectos	Referencias
1-70 µM	9 días	qPRC	La señalización de CREB se reguló a la baja.	Attoff et al., 2020
0, 1,25, 2,5 y 5 mM	24h	Evaluación de citocinas	La vía de señalización NF-κB y MAPKs con JNK y p38 fue activada por el AA.	Yan et al., 2018
2,5 mM	24h	Inmunofluorescencia (hiperfosforilación de tau (pS262))	AA: +50% pS262. Intensidad de fluorescencia de tau fosforilada en el Ser262. La señalización activada de PERK-eIF2α desencadenó la activación de GSK-3β, ATF4 regulado al alza y CHOP. Además, inhibió la activación de la vía PI3K/Akt.	Yan et al., 2022
1,25, 2,5, 5 mM	24h	Western blot	Inhibió la activación de la regulación negativa de la vía PI3K/Akt de la fosforilación de AMPK, Sirt1, PGC-1α y GSK3β por AA de manera dependiente de la dosis; sobreexpresión de COX-2 e iNOS,	Song et al., 2017

AA: Acrylamide, **AMPK:** AMP-activated protein kinase, **COX:** Prostaglandin-endoperoxide synthase, **CREB:** cAMP response element binding, **CHOP:** C/EBP homologous protein, **GSK-3β:** glycogen synthase kinase-3β, **iNOS:** inducible nitric oxide synthase, **JNK:** c-Jun N-terminal kinase, **MAPKS:** mitogen-activated protein kinases, **PERK/eIF2α:** protein kinase RNA-like endoplasmic reticulum kinase - Eukaryotic Initiation Factor 2 alpha, **Pyk2:** proline-rich tyrosine kinase 2 RNA (PKR)-like/Pancreatic ER Kinase, **PI3K:** phosphatidylinositol 3-kinase, **PGC-1α:** Peroxisome proliferator-activated receptor-gamma coactivator, **qPCR:** quantitative polymerase chain reaction, **Sirt 1:** NAD-dependent deacetylase sirtuin-1.

1.10. Expresión génica

1.10.1. Acrilamida (AA)

El impacto de AA en la expresión génica, dilucidado por investigaciones recientes, pone de manifiesto una respuesta compleja y matizada dentro de los sistemas celulares. Un análisis exhaustivo de los genes expresados diferencialmente (DEG) después de la exposición a AA reveló distintos patrones de desregulación génica.

Después de la exposición a 70 µM de AA durante un período de diferenciación de seis días, se observó una desregulación notable en un conjunto de ocho genes. Entre estos genes, NTRK2 (receptor neurotrófico tirosina quinasa 2, un receptor de BDNF), FGF1, RASD2, BMP7, SEMA3F (semáforina 3F) y SEMA5A (semáforina 5A) mostraron alteraciones significativas, lo que indica posibles vías afectadas por la toxicidad inducida por AA durante la diferenciación celular (Hinojosa et al., 2023). En el mismo estudio, también estudiaron otros compuestos; por lo tanto, la desregulación compartida de CNR1 (que codifica el receptor cannabinoide 1) y SEMA5A entre las exposiciones a AA y ácido valproico, así como OPRD1 (que codifica el receptor opioide delta 1) entre AA y rotenona, subraya la intrigante superposición en la respuesta genética entre estos compuestos neurotóxicos (Hinojosa et al., 2023).

Además de examinar los marcadores relacionados con las vías de señalización de CREB, Attoff et al. (2020) optaron por incorporar indicadores asociados a neuronas colinérgicas (CHAT) y dopaminérgicas (DRD2 y MAOA) teniendo en cuenta su papel crucial en el proceso de diferenciación neuronal de las células SH-SY5Y. También incluyeron la sinaptotagmina 1 (SYT1), conocida por su formación de aductos proteicos con AA, junto con genes previamente afectados por la exposición a AA (como MAOA y FGF1). Además, el BDNF y la quinasa receptora de tropomiosina B (TrkB), estrechamente asociados con la vía de señalización CREB, formaron parte de la selección. El análisis reveló una disminución notable en la expresión de 6 de los 16 genes después de la exposición a 70 µM de AA, con 5 de ellos demostrando una disminución de la expresión incluso después de la exposición a 1 µM de AA.

Curiosamente, la expresión de CHAT, TGFB1, STXBP2, BDNF y DRD2 mostró un aumento durante la diferenciación en comparación con las células indiferenciadas. Sin embargo, todos estos genes se regularon a la baja tras la exposición a AA. En particular, si bien la MAOA demostró una mayor expresión en comparación con las células indiferenciadas, solo mostró una reducción significativa después de la exposición a 70 µM de AA (Attoff et al., 2020).

1.10.2. Micotoxinas

En la siguiente sección se abordará la capacidad de alteracion de la expresión genica, de varias micotoxinas relevantes en seguridad alimentaria. Se analizarán específicamente los efectos de DON, FB1, OTA, T-2 y ZEA.

1.10.2.1. Deoxinivalenol (DON)

Para DON, Kalagatur et al. (2021) estudiaron la capacidad de estas micotoxinas para alterar la expresión de marcadores neuronales significativos, reportando una disminución significativa a 50 y 100 µM de la expresión de ARNm de BDNF, AADC y TH.

1.10.2.5. Fumonisina B1 (FB1)

Finalmente, para FB1, se estudió la capacidad de producir daño en el ADN, a través del ensayo COMETA, el ensayo TUNEL y el ensayo de fragmentación del ADN. En el ensayo del COMETA y en comparación con otros tratamientos, el daño en el ADN fue mayor 48 h después del tratamiento con FB1, como lo indica un aumento de la longitud de la cola y una disminución del diámetro de la cabeza. Además, en comparación con el control, un tratamiento con toxinas de 24 horas provocó un daño considerable en el ADN. Además, los resultados del ensayo TUNEL mostraron que el tratamiento con FB1 durante 12, 24 y 48 h aumentó las células TUNEL-positivas en relación con el control (Paul et al., 2021). En concordancia, se observaron escaleras de ADN después del tratamiento con 30 y 100 µM FB1 durante 48 h (Forsby, 2011).

1.10.2.2. Ocratoxina A (OTA)

Del mismo modo, pero a concentraciones más altas de OTA (130-0,20 nM) y a los 7 días de exposición, se encontró una alteración en varios genes del ciclo celular (p21, p53, ciclina B y D) y de diferenciación neuronal (GAP43, Wnt5a y TUBB3) (Frangiamone et al., 2022). En cuanto a la expresión génica y proteica, se estudiaron varios genes y proteínas, todos ellos implicados en la proliferación celular (BAX, P53, MAPT, TPPP p21, ciclina B y D, GAP43, Wnt5a y TUBB3). A la dosis más alta estudiada, la exposición a 1 µM de OTA durante dos días redujo la expresión de P53, BAX y ARNm de MAPT en los días 1 y 2. En comparación con los grupos de exposición de control, la expresión de ARNm de TPPP aumentó el día 2 después de la exposición a 1 µM de OTA, pero disminuyó el día 1 (Sharma et al, 2023). La duración de la exposición y la relación entre la duración de la exposición y la dosis de OTA 1 µM se indicaron mediante la expresión de ARNm de BDNF. Durante 11 días, las células expuestas a dosis menores (2 fM, 20 fM) no mostraron cambios en la expresión génica (Frangiamone et al., 2022).

1.10.2.3. T-2 Toxina

Para investigar el impacto de la toxina T-2 en la biogénesis mitocondrial en células SH-SY5Y, los investigadores midieron el número de copias de ADNmt y los niveles de expresión de NRF2, KEAP1, PGC-1, NRF1 y TFAM (Pang et al., 2022). Para las células tratadas con 5 y 10 ng/mL, el número de copias de ADNmt disminuyó a 80,3% y 60,9%, respectivamente. La expresión del ARNm de NRF2 aumentó a 1,7 y 2,8 veces, respectivamente (Pang et al., 2022). Por el contrario, la expresión de ARNm de KEAP1 cayó al 45,4% y al 77,0%. La toxina T-2 redujo significativamente las expresiones de PGC-1 y sus objetivos posteriores NRF1 y TFAM. Las reducciones más notables se observaron en los niveles de genes, que se redujeron en 5 y 10 ng/mL de toxina T-2, respectivamente, hasta el 56,3% y el 21,6% (Pang et al., 2022).

1.10.2.4. Zearalenona (ZEA) y sus metabolitos

Cuando la concentración de ZEA aumentó en el ensayo del COMETA, la dispersión de la cola aumentó de manera dependiente de la dosis. Además, se observó un aumento sustancial de los núcleos apoptóticos en las células tratadas en comparación con las células control a las 12 y 24 h después de la exposición. Este patrón de "escalera" es indicativo de la fragmentación del ADN oligonucloesómico. Además, la expresión de ARNm de BDNF y TH no se modificó y disminuyó gradualmente con un aumento en la concentración de toxinas durante 12 y 24 h después de la exposición a ZEN cuando se examinó la expresión génica a la dosis más baja de ZEA (25 μM). Por el contrario, a 25 μM, se inhibió la expresión de ARNm de AADC. Se logró una mayor supresión de la expresión de ARNm de AADC mediante el aumento de la concentración de toxina ZEA (50 y 100 μM) (Venkataramana et al., 2014). En el caso de los metabolitos, se estudiaron otros genes y se obtuvieron resultados similares; en las células tratadas con α-ZEL, el ARNm de CASP3 y BAX se sobreexpresó a 12,5 y 25 μM. Y en el caso de β-ZEL, reguló al alza el ARNm de RE a 12,5 μM, mientras que reguló a la baja la expresión de CASP3 y BCL2 (Agahi et al., 2020).

En resumen, las micotoxinas han demostrado la capacidad de modular la expresión génica en las células SH-SY5Y, así como de producir daño en el ADN (Tabla 9).

Tabla 9. *Expresión génica y genotoxicidad en células SH-SY5Y. Dosis de micotoxinas, condiciones de exposición, ensayos y efectos.*

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
DON	0 – 200 µM	6 h y 24 h	Ensayo de cometas Expresión génica por qRT-PCR	DON dañó el ADN de las células SH-SY5Y a las 24 h. La expresión de BDNF y TH mRNA no se alteró con la exposición a 25 µM de DON, pero se reguló significativamente a la baja a 50 y 100 µM. La expresión del gen AADC se reguló a la baja en las tres concentraciones de DON estudiadas.	Kumar Kalagatur N. et al., 2021
BEA	2,5, 1,25, 0,78 y 0,39 µM	2 h, 24 h y 48 h	Ensayo de expresión génica por RT-PCR	BEA reguló al alza el ARNm de BCL2 de manera significativa.	Agahi F et al., 2020b
ZEA α-ZEL y β-ZEL	25, 12.5, 6,25 y 3,12 µM	120 minutos 24 h y 48 h	Ensayo de expresión génica por RT-PCR	α-ZEL: el ARNm de CASP3 y el ARNm de BAX se sobreexpresaron a 12,5 y 25 µM. β-ZEL: ARNm de ERβ regulado al alza a 12,5 µM mientras que la expresión de CASP3 y BCL2 está regulada a la baja.	Agahi F et al., 2020b
	25, 50, 75, 100 y 200 µM	3h, 24h	Ensayo COMET Ensayo de rotura de plásmidos con tinción DAPI Expresión génica por qRT-PCR	La longitud de la cola de la celda de control fue de 10,0 ± 1,5 lm, mientras que en el caso del postratamiento ZEN de 25 µM se midió como 31 ± 2,6, que se incrementó a 83 ± 5,3 con 100 µM ZEN después de la exposición. Las células tratadas con ZEN mostraron fragmentación del ADN a las 12 h y a las 24 h. 25 µM de ZEN: La expresión de BDNF y TH mRNA no se alteró, disminuyó con el aumento de la concentración de la toxina durante 12 y 24 h de exposición a ZEN. La expresión de ARNm de AADC se suprimió a 25 µM. La expresión de ARNm de AADC se anuló aún más con el aumento de la concentración (50 y 100 µM) de ZEN.	M Venkataramana, S. et al., 2014
FB1	1, 10, 100 µM	12 h, 24 h, 48 h, 72 h, 144 h	Fragmentación del ADN	Se observaron escaleras de ADN después del tratamiento con 30 y 100 µM de FB1 durante 48 h.	Stockmann-Juvala H et al 2006

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
	50 µM	12 h, 24 h y 48 h	Ensayo de cometas Ensayo TUNEL:	El tratamiento con FB1 aumentó la longitud de la cola y disminuyó el diámetro de la cabeza en función del tiempo en comparación con el control. Los resultados del ensayo TUNEL revelaron que 12, 24 y 48 h de exposición a FB1 aumentaron las células TUNEL-positivas en comparación con el control.	Souren P et al., 2021
OTA	2 fM, 20 fM y 2 pM 0,001 µM, 0,01 µM, 0,05 µM, 0,1 µM, 0,5 µM, 1 µM	2 y 11 días	Expresión génica por RT-qPCR	La expresión de P53, BAX y ARNm de MAPT se redujo en los días 1 y 2. La expresión de ARNm de TPPP disminuyó en el día 1, pero aumentó en el día 2 de 1 µM OTA. La expresión del ARNm de BDNF mostró una duración de la exposición y una interacción entre la duración de la exposición y la dosis de OTA de 1 µM. 11 días de 2 fM, 20 fM de exposición a OTA, no tuvieron ningún efecto en la expresión de los genes.	Sharma R et al 2023
	0,1, 0,25, 0,5, 1,0 y 2,5 µM	24 h, 48 h	Ensayo de fragmentación del ADN	Se pudo encontrar una escalera de ADN dependiente de la concentración en las células tratadas con OTA, lo que indica que la mayoría de las células pueden sufrir apoptosis.	Zhang X et al 2009
T-2	5 - 20 ng/mL T-2	1 – 48 h	Cuantificación del número de copias de ADN mitocondrial Expresión génica por RT-qPCR	El número de copias del ADNmt se redujo a 80,3% y 60,9%, respectivamente, a 5 y 10 ng/mL. La expresión de ARNm de NRF2 aumentó a 1,7 y 2,8 veces por 5 y 10 ng/ml de toxina T-2, respectivamente. La expresión de ARNm de KEAP1 disminuyó a 77,0% y 45,4%, respectivamente. Las expresiones de PGC-1α y sus dianas posteriores NRF1 y TFAM fueron claramente inhibidas por la toxina T-2. Las disminuciones más prominentes se encontraron en los niveles de genes, que se redujeron a 56,3% y 21,6% por 5 y 10 ng/mL de toxina T-2, respectivamente.	Pang et al., 2022

BAX: Bcl-2 Associated X-protein, **BCL2:** B-cell leukemia/lymphoma 2 protein, **BDNF:** Brain-derived neurotrophic factor, **BEA:** Beauvericin, **CASP3:** cysteine–aspartic acid protease, **DON:** Deoxynivalenol, **ENN'** s: enniatin, **Erβ:** Estrogen receptor beta, **FB1:** Fumonisin B1, **KEAP1:** Kelch-like ECH-associated protein 1, **NRF1:** Nuclear respiratory factor-1, **NRF2:** nuclear factor erythroid 2–related factor 2, **OTA:** Ochratoxin A, **P53:** tumour protein p53, **PGC-1α:** Peroxisome proliferator-activated receptor-gamma coactivator, **qPCR:** quantitative polymerase chain reaction, **TFAM:** Mitochondrial transcription factor A, **ZEA:** Zearalenone, **α-ZEL:** alpha-zearalenol, **β-ZEL:** beta-zearalenol.

1.11. Expresión proteica

1.11.1. Acrilamida (AA)

Si bien el estudio de los factores de transcripción es importante para saber qué vías de señalización pueden ser alteradas, la importancia radica en la expresión final o supresión; es por eso que se consideró el contenido de proteínas y cómo la presencia de AA lo modula.

De acuerdo con las vías de señalización y los factores transcriptómicos alterados como se mencionó anteriormente, se analizaron las proteínas involucradas en los procesos de estrés, diferenciación neuronal, inflamación y muerte celular, como las proteínas de choque térmico (HSPs), las proteínas asociadas a microtúbulos (MAP), la quinasa de adhesión focal (FAK) y CHOP.

En este sentido, por un lado, se ha comprobado que el tratamiento de las células con AA aumentó la muerte celular a través de la actividad de la caspasa-3 y un aumento de las células en la fase sub-G1. Tanto la activación de la caspasa-3 como la población de células sub-G1 alcanzaron su punto máximo cuando las células se expusieron a 3 mM de AA. Curiosamente, una dosis más alta de AA (4-5 mM) resultó en una menor actividad de la caspasa-3, a pesar de que la citotoxicidad aumentó. Esto indica que la disminución de la actividad de la caspasa-3 a 5 mM de AA no se debe únicamente a una mayor citotoxicidad (Okuno et al., 2006).

Un año más tarde, el mismo equipo de investigación descubrió que, después de exponer células de neuroblastoma humano (SH-SY5Y) a 0,5-5 mM de AA durante 18 h, los niveles de HSP de 90, 70 y 27 kDa (Hsp90, Hsp70 y Hsp27, respectivamente) se elevaron en el medio de incubación dependiendo de la dosis de AA, mientras que solo el nivel de Hsp70 aumentó dentro de las células (Zhang et al., 2009).

Según otros trabajos, la exposición a AA hace que el factor de iniciación de la traducción eucariota 2 (eIF2) se fosforile. A esto le sigue la acumulación de ATF4, la

proteína que se une a eIF2. Además, se demostró que la expresión de ARNm de CHOP aumentó drásticamente en todos los puntos temporales y dosis investigados, con un aumento notable de hasta 100 veces a 7,5 o 10 mM AA después de una exposición de 8 h. Por el contrario, no hubo alteración en los niveles proteicos de GRP94, proteína regulada por glucosa (GRP78) o chaperonas del retículo endoplásmico (RE). Estos resultados implican que la exposición a AA de las células SH-SY5Y provoca la producción de la proteína proapoptótica CHOP, pero no de las chaperonas del RE que proporcionan citoprotección (Komoike y Matsuoka, 2016*)*.

Los efectos de AA sobre la proteína p53 y las vías de transducción de señales intracelulares se examinaron utilizando células SH-SY5Y de neuroblastoma humano. p53, p53 fosforilada y la proteína murina doble minuto 2 (MDM2) asociada a p53 fueron reguladas al alza por AA. La posición Ser15 fue la única localización en la que se fosforiló p53. La proteína quinasa (ERK) y p38 reguladas por señales extracelulares fueron fosforiladas por MAPKs a dosis crecientes de AA (0,5-5 mM), pero no c-Jun NH2-terminal quinasa (Okuno et al., 2006)

Numerosas proteínas se ven afectadas por la formación de aductos inducidos por AA, incluida la SNAP-25 (proteína asociada a los sinaptosomas) y otras proteínas que se encuentran en las vesículas sinápticas (LoPachin et al., 2005). Con el fin de confirmar la disminución en la cantidad de axones cuando AA estaba presente, los cocultivos se marcaron con anticuerpos contra la III-tubulina y el marcador panneuronal PGP 9.5 (Sumizawa e Igisu, 2007). El número de perfiles axónicos en respuesta a AA disminuyó en ambos casos, y los resultados coincidieron con la información obtenida de la inmunocitoquímica de SNAP-25.

Según los hallazgos de los estudios in vitro de otros grupos de investigación, la activación de SphK1 en las células SH-SY5Y también controló la señalización de MAPK. Esto incluyó el aumento de la fosforilación de las proteínas quinasas reguladas por señales extracelulares (ERK) y la disminución de JNK y p38 (Frangiamone et al.,

2022). Estos hallazgos implican que la activación de SphK1 puede proporcionar protección a las células nerviosas contra el daño inducido por AA.

Los MAP son responsables de la polimerización, estabilización y dinámica de la red de microtúbulos; se ha detectado que las expresiones de MAP1b y MAP2c en células SH-SY5Y se redujeron en un 39% y 64%, respectivamente, cuando trataron las células con 2 mM de AA (Chen y Chou, 2015), mientras que las expresiones de MAP1b y MAP2c en células U-1240 MG se redujeron en un 52% y 57%, respectivamente. En cuanto a la señalización del transductor de señal de la quinasa Janus (JAK) y del activador de la transcripción (STAT), la expresión de JAK1 aumentó en un 93% en las células SH-SY5Y estimuladas con ácido retinoico (RA) de 10 μM y en un 108% en las células U-1240 MG estimuladas con ácido butírico (BA) de 10 μM. Además, la expresión de JAK1 en las células U-1240 MG se redujo en un 74% cuando se trataron en combinación con 10 μM de BA y 2 mM de AA, mientras que la expresión de JAK1 en las células SH-SY5Y se redujo en un 68% cuando se trataron en combinación con 2 mM de AA y 10 μM de RA. Estos hallazgos demostraron que en las células SH-SY5Y y U-1240 MG, la diferenciación inhibida de A fue mediada por la expresión de MAPs y la señalización de JAK-STAT, argumentando que la exposición a AA inhibe la diferenciación celular en células de neuroblastoma y glioblastoma humanos y que la regulación a la baja de la vía de señal JAK-STAT puede contribuir a la comprensión de la neurodegeneración inducida por AA (Chen y Chou, 2015).

También se ha estudiado el proceso diferenciador de las células SH-SY5Y del neuroblastoma con AA que induce la fosforilación de tirosina dependiente del tiempo de FAK [pY397] y Pyk2[pY402] (Nakagawa-Yagi et al., 2021). En resumen, podemos concluir que las expresiones de MAP1b y MAP2c, típicamente involucradas en las redes de microtúbulos, se redujeron en las células SH-SY5Y y U-1240 MG, lo que indica un claro deterioro del apagado celular tras la exposición a AA. Además, se observó una respuesta proinflamatoria significativa con la sobreexpresión de JAK1. De manera similar, el análisis transcriptómico in vitro mostró cómo la exposición a AA (2,5-5 mM) indujo apoptosis (aumento de los niveles de expresión de p53, MDM2 y caspasa-3),

estrés oxidativo (sobreexpresión de ATP4 y CHOP) e inflamación con altos niveles de IL-6 y TNF-α. También es necesario subrayar la regulación a la baja de SphK1, que se asocia con inflamación y procesos antiapoptóticos (Tabla 10).

Tabla 10. *Expresión de proteínas en células SH-SY5Y. Condiciones de exposición, ensayos y efectos de AA.*

Dosis	Hora	Ensayos	Efectos	Referencias
De 1 a 5 mM	16h y 20h		Aumento de la actividad de la caspasa-3	Sumizawa e Ingisu 2007.
0,5- 5 mM	18h	Análisis de proteínas	Los niveles de Hsp90, 70 y 27 en los medios de incubación aumentaron dependiendo de la dosis de AA. Las PAS aumentan a medida que crece el AA.	Sumizawa e Ingisu 2008
De 1 a 10 mM	8h	qPCR	AA induce la expresión de la proteína proapoptótica CHOP	Komoike y Matsuoka 2016
0,5–5 mM	De 1 a 24 horas	Inmunoprecipitación	La proteína p53 se fosforila en Ser15 y la proteína p53 total se acumula	Okuno et al., 2006
De 0,01 mM a 12 mM.	24, 96 o 144 h	Inmunotransferencia	No hubo cambios en la proteína SNAP-25 con concentraciones de AA de 0,5 mM, el análisis de imágenes mostró que hubo una disminución progresiva con la exposición a 1 mM de AA y superior. (0,5-2,0 mM) se asociaron con una reducción del 30% en los perfiles axónicos positivos para SNAP-25.	Lourenssen et al., 2009
0, 0,1, 0,5, 1 y 2 mM	24, 48, 72h	Western blot	La expresión de MAPs y la señalización de JAK-STAT estuvieron implicadas en la diferenciación inhibida por AA	Chen y Chou 2015
6 mM	8h	Análisis de pernos occidentales y PCR	Fosforilación de tirosina dependiente del tiempo de FAK [pY397] y Pyk2 [pY402]	Nakagawa-Yagi et al., 2001

AA: Acrylamide, , **CHOP:** C/EBP homologous protein, **FAK:** focal adhesion kinase, **HSPs:** Heat shock protein, **IC_{20}:** concentration causing 20% protein reduction, **IC_{50}:** 50%inhibition concentration, **JAK-STAT:** janus kinase- signal transducer and activator of transcription, **PERK:** protein kinase, **Pyk2:** proline-rich tyrosine kinase 2 RNA (PKR)-like/Pancreatic ER Kinase, **qPCR:** quantitative Polymerase chain reaction, **SNAP:** synaptic vesicle protein S-nitroso-N-acetyl penicillamine

1.11.2. Micotoxinas (FB1, OTA, T-2 and ZEA)

En la siguiente sección se abordará la capacidad de alteracion de la expresión proteica, de varias micotoxinas relevantes en seguridad alimentaria. Se analizarán específicamente los efectos de FB1, OTA, T-2 y ZEA.

1.11.2.1. Fumonisin B1 (FB1)

Otros estudios han evaluado la capacidad de FB1 para inducir apoptosis, por lo que algunos autores investigaron las alteraciones en la expresión de proteínas (caspasa 9 y 3, p53, Bax, Bcl-2, Bcl-XL y Mcl-1) cuando se aplicó FB1 a células SH-SY5Y. Inicialmente, la exposición a FB1 no dio lugar a alteraciones significativas en la actividad de la proteasa similar a la caspasa-3. Sin embargo, después de un día completo, 100 µM FB1 causaron un aumento leve pero estadísticamente insignificante en la actividad de la caspasa-3. Durante ninguno de los períodos de tratamiento con FB1, no hubo escisión de caspasa-3 o caspasa-9. La exposición a 100 µM de FB1 durante 72 h tampoco afectó a las cantidades de p53 en los extractos nucleares o citoplasmáticos. Estos hallazgos son consistentes con investigaciones anteriores que no descubrieron un aumento estadísticamente significativo en la mortalidad celular a las concentraciones examinadas (0,1-30 µM) (Pérez-Fuentes et al., 2021; Paul et al., 2021; Forsby, 2011*).*

Por otro lado, en comparación con las células control, FB1 no tuvo ningún efecto sobre la expresión de la proteína proapoptótica Bax en ninguna de las dosis o puntos temporales. Además, en las células SH-SY5Y, FB1 no tuvo ningún efecto sobre las proteínas antiapoptóticas investigadas (Bcl-2, Bcl-XL y Mcl-1) (Stockmann-Juvala et al., 2006). Otro estudio sugirió que las mitocondrias pueden estar implicadas en la muerte celular mediada por FB1 porque la expresión de Bax aumentó 48 h después del tratamiento con FB1 en comparación con el control, mientras que la expresión del citocromo C aumentó 24 h después del tratamiento con FB1 (Pérez-Fuentes et al., 2021). Después de la exposición de las células de neuroblastoma a FB1, hubo un aumento significativo en la fosforilación de JNK, que alcanzó su punto máximo a las

48 h. Además, hubo un aumento significativo en la expresión de IRE1-α y PERK. Por último, hubo una activación significativa de CHOP después de 24 h de exposición a FB1 (Paul et al., 2021).

1.11.2.2. Ocratoxina A (OTA)

El tratamiento con OTA en células SH-SY5Y activó la caspasa-9 y la caspasa-3 en un estudio, mientras que en otro estudio no se detectaron caspasas-3 activadas ni cambios en la fosforilación de p53. Esto sugiere que la citotoxicidad inducida por OTA puede tener diferentes mecanismos en juego (Yoon et al., 2009; Nakagawa-Yagi et al., 2001).

1.11.2.3. T-2 Toxina

En cuanto a la toxina T-2, condujo a un aumento en la expresión de la proteína NRF2, lo que podría indicar una respuesta adaptativa al estrés oxidativo inducido por la toxina (Pang et al., 2022).

En cuanto a las micotoxinas, FB1 aumentó significativamente la fosforilación de JNK en células de neuroblastoma, así como aumentó significativamente la expresión de CHOP, IRE1- y PERK. El tratamiento con OTA en SH-SY5Y activó la caspasa-9 y la caspasa-3 en un estudio, pero no en otro. Finalmente, la toxina T-2 aumentó la expresión de la proteína NRF2. Por lo tanto, se necesitan más estudios para afirmar que FB1, OTA y la toxina T-2 condujeron a una modulación en la expresión de proteínas en las células SH-SY5Y (Tabla 11).

Tabla 11. *Expresión de proteínas en células SH-SY5Y. Dosis de micotoxinas, condiciones de exposición, ensayos y efectos.*

Micotoxina	Dosis	Tiempo de exposición	Ensayos	Efectos	Referencias
FB1	1, 10, 100 µM	12 h, 24 h, 48 h, 72 h, 144 h	Actividad de la proteasa similar a la caspasa-3 Western blot	FB1 no causó cambios significativos en la actividad de la proteasa similar a la caspasa-3. 100 µM de FB1 durante 72 h no tuvieron efectos sobre los niveles de p53. Bax Bcl-2, Bcl-XL y Mcl-1 no se vieron afectados por FB1.	Stockmann-Juvala H et al 2006
OTA	OTA de 1, 10 o 100 µM	30, 60 min, 24 h	Western blot	No se detectó caspasa-3 activada. No se detectaron cambios en la fosforilación de p53.	Yoon S et al., 2009
	OTA de 1, 10 o 100 µM	30, 60 min, 24 h	Western blot	No se detectó caspasa-3 activada. No se detectaron cambios en la fosforilación de p53.	Yoon S et al., 2009
	0,1, 0,25, 0,5, 1,0 y 2,5 µM	24 h, 48 h	Western blot	Se activaron la caspasa-9 y la caspasa-3. Z-VAD-fmk y Z-DEVD-fmk fueron eficaces en la prevención de la toxicidad celular inducida por OTA.	Zhang X et al 2009
T-2	5 - 20 ng/mL T-2	1 – 48 h	Western blot	La expresión de la proteína NRF2 se incrementó a 1,2 y 1,4 veces con 5 y 10 ng/mL de toxina T-2, respectivamente. La expresión de la proteína ARNm disminuyó a 95,6% y 85,6%, respectivamente. Las disminuciones más prominentes se encontraron cuando los niveles de proteína disminuyeron a 84.8% y 60.2% por 5 y 10 ng/mL de toxina T-2, respectivamente.	Pang et al., 2022

BAX: Bcl-2 Associated X-protein, **BEA:** Beauvericin, **DON:** Deoxynivalenol, **ENN'** s: enniatin, **FB1:** Fumonisin B1, **NRF2:** nuclear factor erythroid 2–related factor 2, **OTA:** Ochratoxin A, **P53:** tumor protein p53, **ZEA:** Zearalenone, **Z-VAD-fmk:** carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone, **Z- DEVD-fmk:** Z-D(OMe)E(Ome)VD(OMe)-FMK. Z-DEVD-fluoromethylketone. Z-DEVD-fluoromethyl ketone. specific inhibitor of caspase-3, **α-ZEL:** alpha-zearalenol, **β-ZEL:** beta-zearalenol.

1.12. Compuestos bioactivos naturales

Una vez establecido que la AA y las micotoxinas producen toxicidad en la linea celular SH-SY5Y de forma dependiente de la dosis y el tiempo de exposición, diversos estudios han señalado que ciertos compuestos naturales podrían atenuar estos efectos adversos, actuando como agentes neuroprotectores frente al daño inducido por la AA y las micotoxinas. Se encontró que 2 h de pretratamiento con 6 μM de curcumina reduce considerablemente la toxicidad neuronal causada por 2,5 mM de AA. Esto se demostró mediante la mejora de la viabilidad celular, la reducción de los niveles de ROS y MDA intracelulares y el aumento de los niveles de GSH. Además, el pretratamiento con curcumina eliminó la fosforilación aberrante de tau, la disminución de P-CREB y la apoptosis inducida por CHOP en las células SH-SY5Y, disminuyó aún más la actividad de GSK-3 y ATF4 y bloqueó la fosforilación de eIF2 dependiente de PERK (Yan et al., 2022). En esta misma línea, pero con otro compuesto, Chen y Chou (2015) demostraron que 0,25, 0,5 y 1 mM de cafeína atenuaban la fosforilación inhibida por 2 mM de AA de MAPKs en células U-1240 MG. Además, 50 μM de Z-VAD-fmk, un inhibidor de la pancaspasa, podrían ser un compuesto protector contra 0 a 5 mM de AA al abolir las actividades de la caspasa-3 en las células expuestas a AA, disminuyendo la fuga de LDH y aumentando la viabilidad celular (Hartley et al., 1997).

Profundizando en esta cuestión y con el fin de obtener resultados más precisos, Ning et al. (2021), demostraron que de 0,014 a 10 μg/mL de triphala, una mezcla de hierbas de la India, suprimieron la neurotoxicidad inducida por 10 mM AA y eliminaron los radicales libres en un modelo de pez cebra, lo que sugiere que la triphala podría ser un agente potencial para tratar enfermedades neurodegenerativas asociadas con el estrés oxidativo (Tabla 12).

En cuanto a las micotoxinas y las posibles estrategias para mitigar su citotoxicidad, varios estudios han reportado la capacidad citoprotectora de algunos compuestos bioactivos, como el ajo (Allium sativum L.) frente a los metabolitos BEA y ZEA (Agahi et al., 2022), mientras que los subproductos del café han demostrado efectos similares

frente a la BEA (Juan et al., 2020), la NAC frente a FB1 (Paul et al., 2021), la luteína, la zeaxantina y el extracto de bayas de goji frente a la BEA (Montesano et al., 2020), y extracto de remolacha frente a FB1 y OTA (Penalva-Olcina et al., 2022) (Tabla 12 y Figura 3).

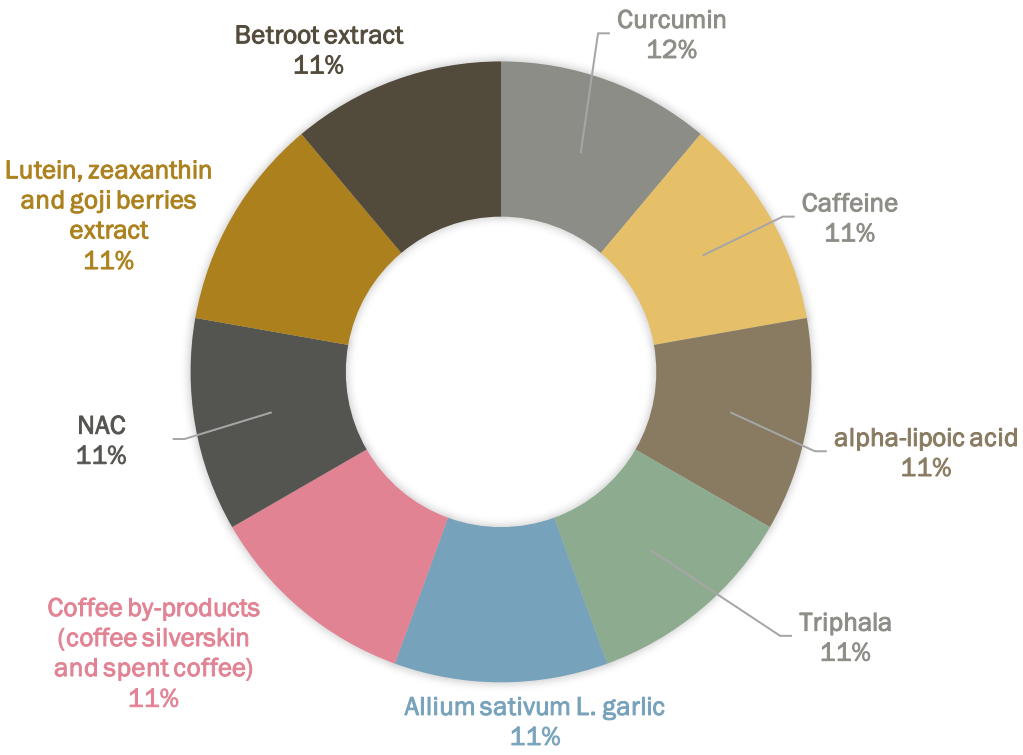


Figura 3. *Gráfico del porcentaje de compuestos bioactivos probados en los artículos seleccionados (n=9).*

Tabla 12. Dosis de compuestos bioactivos naturales, tiempo de pretratamiento, compuesto y efecto.

	Dosis	Hora	Compuesto	Efectos	Referencias
AA	6 µM de	2 h de pretratamiento	Curcumina	Aumento de la viabilidad de la célula La curcumina alivia el estrés oxidativo inducido por AA: GRP78, P-PERK y P-eIF2α disminuyeron significativamente en las células pretratadas con curcumina La curcumina bloquea la señalización PERK-eIF2α causada por AA Activación La curcumina suprime la tau inducida por AA Hiperfosforilación La curcumina mitiga la apoptosis neuronal inducida por AA	Yan et al., 2022
	0, 0,25, 0,5 y 1 mM	30 min de pretratamiento	Cafeína	Fosforilación inhibida por AA atenuada por cafeína de MAPKs en U-1240 MG células.	Chen y Chou 2015
	50 µM	1h	Z-VAD-fmk	Z-VAD-fmk abolió las actividades de la caspasa-3 en las células expuestas a AA, disminuyó la fuga de LDH y aumentó la viabilidad celular.	Sumizawa e Ingisu 2007
	0,123, 0,370, 1,11, 3,33 y 10,0 µg/mL	20h	Triphala	Triphala disminuyó significativamente el nivel de radicales libres producidos por AA. Triphala tiene efectos neuroprotectores *in vivo* contra AA en el modelo de pez cebra.	Ning et al., 2021
BEA, α-ZEL y β-ZEL		24 h y 48 h	*Allium sativum L.* ajo	La viabilidad de la célula aumentó significativamente	Agahi et al., 2022
BEA		24 h y 48 h	Subproductos del café (café plateado y café usado)	Citoprotección	Juan et al., 2020
FB1		24h	NAC	La generación de ROS inducida por FB1 se anuló fuertemente cuando se trató un eliminador de ROS, N-acetil-L-cisteína (NAC), durante 24 h	Souren et al., 2021

	Dosis	Hora	Compuesto	Efectos	Referencias
BEA			Extracto de luteína, zeaxantina y bayas de goji	Citoprotección	Montesano et al., 2020
FB1 y OTA		24h	*Extracto de Betroot*	Los resultados revelaron un aumento en la viabilidad celular para casi todas las concentraciones ensayadas, siendo las mayores a [50 + 25] µM, [25 + 12.5] µM y [12.5 + 6.25] µM con 118%, 94% y 104%, respectivamente	Penalva-Olcina et al., 2022

AA: Acrylamide, **eIF2α:** Eukaryotic Initiation Factor 2 alpha, **GRP78:** glucose regulated protein 78, **LDH:** lactate dehydrogenase, **MAPKs:** mitogen-activated protein kinases, **P:** Phospho, **PERK:** protein kinase, **Pyk2:** proline-rich tyrosine kinase 2 RNA (PKR)-like/Pancreatic ER Kinase, **U-1240 MG:** Human glioblastoma astrocytoma cells. **Z-VAD-fmk:** Z-Val-Ala-Asp (OMe)-FM

1.13. Bibliografia

Abudayyak, M.; Karaman, E.F.; Ozden, S. Mechanisms Underlying Citrinin-Induced Toxicity via Oxidative Stress and Apoptosis- Mediated by Mitochondrial-Dependent Pathway in SH-SY5Y Cells. Drug Chem. Toxicol. 2022, 46, 944–954.

Acrylamide (IARC Summary & Evaluation, Volume 60, 1994). Available online: https://inchem.org/documents/iarc/vol60/m6 0-11.html (accessed on 29 June 2023).

Agahi, F.; Álvarez-Ortega, N.; Font, G.; Juan-García, A.; Juan, C. Oxidative Stress, Glutathione, and Gene Expression as Key Indicators in SH-SY5Y Cells Exposed to Zearalenone Metabolites and Beauvericin. Toxicol. Lett. 2020, 334, 44–52.

Agahi, F.; Font, G.; Juan, C.; Juan-García, A. Individual and Combined Effect of Zearalenone Derivates and Beauvericin Mycotoxins on SH-SY5Y Cells. Toxins 2020, 12, 212.

Agahi, F.; Juan-García, A.; Font, G.; Juan, C. Study of Enzymatic Activity in Human Neuroblastoma Cells SH-SY5Y Exposed to Zearalenone's Derivates and Beauvericin. Food Chem. Toxicol. 2021, 152, 112227.

Agahi, F.; Juan, C.; Font, G.; Juan-García, A. Neurotoxicity of Zearalenone's Metabolites and Beauvericin Mycotoxins via Apoptosis and Cell Cycle Disruption. Toxicology 2021, 456, 152784.

Agahi, F.; Penalva-Olcina, R.; Font, G.; Juan-García, A.; Juan, C. Effects of Voghiera Garlic Extracts in Neuronal Human Cell Line against Zearalenone's Derivates and Beauvericin. Food Chem. Toxicol. 2022, 162, 112905.

All News—ECHA. Available online: https://echa.europa.eu/-/one-hazardous-chemical-added-to-the-candidate-list (accessed on 29 June 2023).

Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ, Schinkel AH., 2002. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther. Apr;1(6):417-25. PMID: 12477054.

Anese, M. Acrylamide in coffee and coffee substitutes. In Acrylamide in Food: Analysis, Content and Potential Health Effects; Academic Press Elsevier: Waltham, MA, USA, 2016.

Arp, L.H., Richard, J.L., 1981. Experimental intoxication of guinea pigs with multiple doses of the mycotoxin, penitrem A. Mycopathologia 73, 109–113. https://doi.org/10.1007/BF00562600/METRICS

Attoff, K.; Johansson, Y.; Cediel-Ulloa, A.; Lundqvist, J.; Gupta, R.; Caiment, F.; Gliga, A.; Forsby, A. Acrylamide Alters CREB and Retinoic Acid Signalling Pathways during Differentiation of the Human Neuroblastoma SH-SY5Y Cell Line. Sci. Rep. 2020, 10, 16714.

Attoff, K.; Kertika, D.; Lundqvist, J.; Oredsson, S.; Forsby, A. Acrylamide Affects Proliferation and Differentiation of the Neural Progenitor Cell Line C17.2 and the Neuroblastoma Cell Line SH-SY5Y. Toxicol. Vitr. 2016, 35, 100–111.

Ban, M.; Shimoda, R.; Chen, J. Investigation of Nanoplastic Cytotoxicity Using SH-SY5Y Human Neuroblastoma Cells and Polystyrene Nanoparticles. Toxicol. Vitr. 2021, 76, 105225.

Berntsen, H.F., Bogen, I.L., Wigestrand, M.B., Fonnum, F., Walaas, S.I., Moldes-Anaya, A., 2017. The fungal neurotoxin penitrem A induces the production of reactive oxygen species in human neutrophils at submicromolar concentrations. Toxicology 392, 64–70. https://doi.org/10.1016/J.TOX.2017.10.008

Boysen, S.R., Rozanski, E.A., Chan, D.L., Grobe, T.L., Fallon, M.J., Rush, J.E., 2002. Tremorgenic mycotoxicosis in four dogs from a single household. J Am Vet Med Assoc 221, 1441–1444. https://doi.org/10.2460/JAVMA.2002.221.1441

Breton, P., Bizot, J.C., Buee, J., De La Manche, I., 1998. Brain neurotoxicity of Penitrem A: electrophysiological, behavioral and histopathological study. Toxicon 36, 645–655. https://doi.org/10.1016/S0041-0101(97)00084-6

Bridgeman, L.; Juan, C.; Juan-García, A.; Berrada, H. Individual and Combined Effect of Acrylamide, Fumitremorgin C and Penitrem A on Human Neuroblastoma SH-SY5Y Cells. Food Chem. Toxicol. 2023, 182, 278–6915.

Calcagno, A.M., Fostel, J.M., To, K.K.W., Salcido, C.D., Martin, S.E., Chewning, K.J., Wu, C.P., Varticovski, L., Bates, S.E., Caplen, N.J., Ambudkar, S. V., 2008. Single-step doxorubicin-selected cancer cells overexpress the ABCG2 drug transporter through epigenetic changes. British Journal of Cancer 2008 98:9 98, 1515–1524. https://doi.org/10.1038/sj.bjc.6604334

Catovic, S., Filipovic, N., Stern, P., 1975. The effect of penitrem-A upon the level of glycine in the CNS.

Cavanagh, J.B., Holton, J.L., Nolan, C.C., Ray, D.E., Naik, J.T., Mantle, P.G., 1998. The effects of the tremorgenic mycotoxin penitrem A on the rat cerebellum. Vet Pathol 35, 53–63. https://doi.org/10.1177/030098589803500105

Chen, J.H.; Chou, C.C. Acrylamide Inhibits Cellular Differentiation of Human Neuroblastoma and Glioblastoma Cells. Food Chem. Toxicol. 2015, 82, 27–35.

Chen, J.H.;Wu, K.Y.; Chiu, I.M.; Tsou, T.C.; Chou, C.C. Acrylamide-Induced Astrogliotic and Apoptotic Responses in Human Astrocytoma Cells. Toxicol. Vitr. 2009, 23, 855–861.

De Boevre, M., Di Mavungu, J.D., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., De Saeger, S., 2012. Natural occurrence of mycotoxins and their masked forms in food and feed products. World Mycotoxin J 5, 207–219. https://doi.org/10.3920/WMJ2012.1410

Doi, K.; Uetsuka, K. Mechanisms of Mycotoxin-Induced Neurotoxicity through Oxidative Stress-Associated Pathways. Int. J. Mol. Sci. 2011, 12, 5213–5237.

Domijan, A.M.; Abramov, A.Y. Fumonisin B1 Inhibits Mitochondrial Respiration and Deregulates Calcium Homeostasis-- Implication to Mechanism of Cell Toxicity. Int. J. Biochem. Cell Biol. 2011, 43, 897–904.

Eisenbrand, G. Revisiting the Evidence for Genotoxicity of Acrylamide (AA), Key to Risk Assessment of Dietary AA Exposure. Arch. Toxicol. 2020, 94, 2939–2950.

El-Sayed, R.A.; Jebur, A.B.; Kang,W.; El-Demerdash, F.M. An Overview on the Major Mycotoxins in Food Products: Characteristics, Toxicity, and Analysis. J. Future Foods 2022, 2, 91–102.

EUR-Lex—32017R2158—EN—EUR-Lex. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX: 32017R2158 (accessed on 29 June 2023).

Evans, T.J., Gupta, R.C., 2018. Tremorgenic Mycotoxins. Veterinary Toxicology: Basic and Clinical Principles: Third Edition 1033–1041. https://doi.org/10.1016/B978-0-12-811410-0.00074-X

Exon, J.H. A Review of the Toxicology of Acrylamide. J. Toxicol. Environ. Health B Crit. Rev. 2006, 9, 397–412.

Forsby, A. Neurite Degeneration in Human Neuronal SH-SY5Y Cells as an Indicator of Axonopathy. Neuromethods 2011, 56, 255–268.

Frangiamone, M.; Alonso-Garrido, M.; Font, G.; Cimbalo, A.; Manyes, L. Pumpkin Extract and Fermented Whey Individually and in Combination Alleviated AFB1- and OTA-Induced Alterations on Neuronal Differentiation in Vitro. Food Chem. Toxicol. 2022, 164, 113011.

Friedman, M.; Mottram, D. Chemistry and Safety of Acrylamide in Food; Springer: New York, NY, USA, 2005; p. 561.

Frimat, J.P.; Sisnaiske, J.; Subbiah, S.; Menne, H.; Godoy, P.; Lampen, P.; Leist, M.; Franzke, J.; Hengstler, J.G.; Van Thriel, C.; et al. The Network Formation Assay: A Spatially Standardized Neurite Outgrowth Analytical Display for Neurotoxicity Screening. Lab Chip 2010, 10, 701–709.

Ghanayem, B.I.; McDaniel, L.P.; Churchwell, M.I.; Twaddle, N.C.; Snyder, R.; Fennell, T.R.; Doerge, D.R. Role of CYP2E1 in the Epoxidation of Acrylamide to Glycidamide and Formation of DNA and Hemoglobin Adducts. Toxicol. Sci. 2005, 88, 311–318.

Guenther, H.; Anklam, E.;Wenzl, T.; Stadler, R.H. Acrylamide in Coffee: Review of Progress in Analysis, Formation and Level Reduction. Food Addit. Contam. 2007, 24, 60–70.

Gupta, R.; Nanda, S.J. Cloud detection in satellite images with classical and deep neural network approach: A review. Multimed. Tools Appl. 2022, 81, 31847–31880.

Hallier-Vanuxeem, D.; Prieto, P.; Culot, M.; Diallo, H.; Landry, C.; Tähti, H.; Cecchelli, R. New Strategy for Alerting Central Nervous System Toxicity: Integration of Blood–Brain Barrier Toxicity and Permeability in Neurotoxicity Assessment. Toxicol. Vitr. 2009, 23, 447–453.

Hartley, C.L.; Anderson, V.E.R.; Anderson, B.H.; Robertson, J. Acrylamide and 2,5-Hexanedione Induce Collapse of Neurofilaments in SH-SY5Y Human Neuroblastoma Cells to Form Perikaryal Inclusion Bodies. Neuropathol. Appl. Neurobiol. 1997, 23, 364–372.

Hinojosa, M.G.; Johansson, Y.; Cediel-Ulloa, A.; Ivanova, E.; Gabring, N.; Gliga, A.; Forsby, A. Evaluation of MRNA Markers in Differentiating Human SH-SY5Y Cells for Estimation of Developmental Neurotoxicity. Neurotoxicology 2023, 97, 65–77.

Hocking, A.D., Holds, K., Tobin, N.F., 1988. Intoxication by tremorgenic mycotoxin (penitrem A) in a dog. Aust Vet J 65, 82–85. https://doi.org/10.1111/j.1751-0813.1988.tb07366.x

Iqbal, S.Z. Mycotoxins in food, recent development in food analysis and future challenges; a review. Curr. Opin. Food Sci. 2021, 42, 237–247.

IRIS Toxicological Review of Acrylamide (External Review Draft) |Risk Assessment Portal| US EPA. Available online: https: //cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=187729 (accessed on 8 January 2024).

Juan-García, A., Juan, C., König, S., Ruiz, M.J., 2015. Cytotoxic effects and degradation products of three mycotoxins: Alternariol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol in liver hepatocellular carcinoma cells. Toxicol Lett 235, 8–16. https://doi.org/10.1016/J.TOXLET.2015.03.003

Juan-García, A.; Caprioli, G.; Sagratini, G.; Mañes, J.; Juan, C. Coffee Silverskin and Spent Coffee Suitable as Neuroprotectors against Cell Death by Beauvericin and α-Zearalenol: Evaluating Strategies of Treatment. Toxins 2021, 13, 132.

Juan, C.; de Simone, G.; Sagratini, G.; Caprioli, G.; Mañes, J.; Juan-García, A. Reducing the Effect of Beauvericin on Neuroblastoma SH-SY5Y Cell Line by Natural Products. Toxicon 2020, 188, 164–171.

Kalagatur, N.K.; Abd_Allah, E.F.; Poda, S.; Kadirvelu, K.; Hashem, A.; Mudili, V.; Siddaiah, C. Quercetin Mitigates the Deoxynivalenol Mycotoxin Induced Apoptosis in SH-SY5Y Cells by Modulating the Oxidative Stress Mediators. Saudi J. Biol. Sci. 2021, 28, 465–477.

Kalinina, S.A., Jagels, A., Cramer, B., Geisen, R., Humpf, H.U., 2017. Influence of Environmental Factors on the Production of Penitrems A–F by Penicillium crustosum. Toxins (Basel) 9, 210. https://doi.org/10.3390/TOXINS9070210

Kalinovskii, A.P.; Osmakov, D.I.; Koshelev, S.G.; Lubova, K.I.; Korolkova, Y.V.; Kozlov, S.A.; Andreev, Y.A. Retinoic Acid- Differentiated Neuroblastoma SH-SY5Y Is an Accessible In Vitro Model to Study Native Human Acid-Sensing Ion Channels 1a (ASIC1a). Biology 2022, 11, 167.

Komoike, Y.; Matsuoka, M. Endoplasmic Reticulum Stress-Mediated Neuronal Apoptosis by Acrylamide Exposure. Toxicol. Appl. Pharmacol. 2016, 310, 68–77.

Kopan´ ska, M.; Łagowska, A.; Kuduk, B.; Banas´-Za¸bczyk, A. Acrylamide Neurotoxicity as a Possible Factor Responsible for Inflammation in the Cholinergic Nervous System. Int. J. Mol. Sci. 2022, 23, 2030.

Leite, M.; Freitas, A.; Barbosa, J.; Ramos, F. Comprehensive Assessment of Different Extraction Methodologies for Optimization and Validation of an Analytical Multi-Method for Determination of Emerging and Regulated Mycotoxins in Maize by UHPLCMS/ MS. Food Chem. Adv. 2023, 2, 100145.

Lewis, P.R., Donoghue, M.B., Hocking, A.D., Cook, L., Granger, L. V., 2005. Tremor syndrome associated with a fungal toxin: sequelae of food contamination. Med J Aust 182, 582–584. https://doi.org/10.5694/J.1326-5377.2005.TB06819.X

Lineback, D.R.; Coughlin, J.R.; Stadler, R.H. Acrylamide in Foods: A Review of the Science and Future Considerations. Annu. Rev. Food Sci. Technol. 2012, 3, 15–35.

LoPachin, R.M. Acrylamide Neurotoxicity: Neurological, Morhological and Molecular Endpoints in Animal Models. Adv. Exp. Med. Biol. 2005, 561, 21–37.

LoPachin, R.M.; Gavin, T. Molecular Mechanism of Acrylamide Neurotoxicity: Lessons Learned from Organic Chemistry. Environ. Health Perspect. 2012, 120, 1650–1657. [CrossRef] [PubMed]

Lourenssen, S.; Miller, K.G.; Blennerhassett, M.G. Discrete Responses of Myenteric Neurons to Structural and Functional Damage by Neurotoxins in Vitro. Am. J. Physiol. Gastrointest Liver Physiol. 2009, 297, G228–G239.

Michalak, J.; Czarnowska-Kujawska, M.; Klepacka, J.; Gujska, E. Zbieta Effect of Microwave Heating on the Acrylamide Formation in Foods. Molecules 2020, 25, 4140.

Moldes-Anaya, A., Wilkins, A.L., Rundberget, T., Fæste, C.K., 2009. In vitro and in vivo hepatic metabolism of the fungal neurotoxin penitrem A. Drug Chem Toxicol 32, 26–37. https://doi.org/10.1080/01480540802416232

Montesano, D.; Juan-García, A.; Mañes, J.; Juan, C. Chemoprotective Effect of Carotenoids from Lycium Barbarum L. on SH-SY5Y Neuroblastoma Cells Treated with Beauvericin. Food Chem. Toxicol. 2020, 141, 111414.

Munday, J.S., Thompson, D., Finch, S.C., Babu, J. V., Wilkins, A.L., di Menna, M.E., Miles, C.O., 2008. Presumptive tremorgenic mycotoxicosis in a dog in New Zealand, after eating mouldy walnuts. N Z Vet J 56, 145–147. https://doi.org/10.1080/00480169.2008.36823

Murtaza, B., Wang, L., Li, X., Nawaz, M.Y., Saleemi, M.K., Khatoon, A., Yongping, X., 2024. Recalling the reported toxicity assessment of deoxynivalenol, mitigating strategies and its toxicity mechanisms: Comprehensive review. Chem Biol Interact 387, 110799. https://doi.org/10.1016/J.CBI.2023.110799

Nakagawa-Yagi, Y.; Choi, D.K.; Ogane, N.; Shimada, S.I.; Seya, M.; Momoi, T.; Ito, T.; Sakaki, Y. Discovery of a Novel Compound: Insight into Mechanisms for Acrylamide-Induced Axonopathy and Colchicine-Induced Apoptotic Neuronal Cell Death. Brain Res. 2001, 909, 8–19.

Ning, W.; Li, S.; Tsering, J.; Ma, Y.; Li, H.; Ma, Y.; Ogbuehi, A.C.; Pan, H.; Li, H.; Hu, S.; et al. Protective Effect of Triphala against Oxidative Stress-Induced Neurotoxicity. Biomed. Res. Int. 2021, 2021, 6674988.

Nordin-Andersson, M.; Forsby, A.; Heldring, N.; DeJongh, J.; Kjellstrand, P.;Walum, E. Neurite Degeneration in Differentiated Human Neuroblastoma Cells. Toxicol. Vitr. 1998, 12, 557–560.

Nordin-Andersson, M.; Walum, E.; Kjellstrand, P.; Forsby, A. Acrylamide-Induced Effects on General and Neurospecific Cellular Functions during Exposure and Recovery. Cell Biol. Toxicol. 2003, 19, 43–51.

Norris, P.J., Smith, C.C.T., De Belleroche, J., Bradford, H.F., Mantle, P.G., Thomas, A.J., Penny, R.H.C., 1980. Actions of tremorgenic fungal toxins on neurotransmitter release. J Neurochem 34, 33–42. https://doi.org/10.1111/J.1471-4159.1980.TB04618.X

Nowak, A.; Zakłos-Szyda, M.; Zyzelewicz, D.; Koszucka, A.; Motyl, I. Acrylamide Decreases Cell Viability, and Provides Oxidative Stress, DNA Damage, and Apoptosis in Human Colon Adenocarcinoma Cell Line Caco-2. Molecules 2020, 25, 368.

Okuno, T.; Matsuoka, M.; Sumizawa, T.; Igisu, H. Involvement of the Extracellular Signal-Regulated Protein Kinase Pathway in Phosphorylation of P53 Protein and Exerting Cytotoxicity in Human Neuroblastoma Cells (SH-SY5Y) Exposed to Acrylamide. Arch. Toxicol. 2006, 80, 146–153.

Pang, Y.; Zhang, L.; Liu, Q.; Peng, H.; He, J.; Jin, H.; Su, X.; Zhao, J.; Guo, J. NRF2/PGC-1 -Mediated Mitochondrial Biogenesis Contributes to T-2 Toxin-Induced Toxicity in Human Neuroblastoma SH-SY5Y Cells. Toxicol. Appl. Pharmacol. 2022, 451, 116167.

Pasquali, M., Beyer, M., Logrieco, A., Audenaert, K., Balmas, V., Basler, R., Boutigny, A.L., Chrpová, J., Czembor, E., Gagkaeva, T., González-Jaén, M.T., Hofgaard, I.S., Köycü, N.D., Hoffmann, L., Levic, J., Marin, P., Miedaner, T., Migheli, Q., Moretti, A., Müller, M.E.H., Munaut, F., Parikka, P., Pallez-Barthel, M., Piec, J., Scauflaire, J., Scherm, B., Stankovic, S., Thrane, U., Uhlig, S., Vanheule, A., Yli-Mattila, T., Vogelgsang, S., 2016. A European database of Fusarium graminearum and F. culmorum trichothecene genotypes. Front Microbiol 7, 182512. https://doi.org/10.3389/FMICB.2016.00406/BIBTEX

Paul, S.; Jakhar, R.; Bhardwaj, M.; Chauhan, A.K.; Kang, S.C. Fumonisin B1 Induces Poly (ADP-Ribose) (PAR) Polymer-Mediated Cell Death (Parthanatos) in Neuroblastoma. Food Chem. Toxicol. 2021, 154, 112326.

Penalva-Olcina, R.; Juan, C.; Fernández-Franzón, M.; Juan-García, A. Effectiveness of Beetroot Extract in SH-SY5Y Neuronal Cell Protection against Fumonisin B1, Ochratoxin A and Its Combination. Food Chem. Toxicol. 2022, 165, 113164.

Pérez-Fuentes, N.; Alvariño, R.; Alfonso, A.; González-Jartín, J.; Gegunde, S.; Vieytes, M.R.; Botana, L.M. Single and Combined Effects of Regulated and Emerging Mycotoxins on Viability and Mitochondrial Function of SH-SY5Y Cells. Food Chem. Toxicol. 2021, 154, 112308.

Peterson, D.W., Penny, R.H.C., Day, J.B., Mantle, P.G., 1982. A comparative study of sheep and pigs given the tremorgenic mycotoxins verruculogen and penitrem A. Res Vet Sci 33, 183–187. https://doi.org/10.1016/S0034-5288(18)32332-4

Pinton, P., Oswald, I.P., 2014. Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. Toxins 2014, Vol. 6, Pages 1615-1643 6, 1615–1643. https://doi.org/10.3390/TOXINS6051615

Popova, D.; Karlsson, J.; Jacobsson, S.O.P. Comparison of Neurons Derived from Mouse P19, Rat PC12 and Human SH-SY5Y Cells in the Assessment of Chemical- and Toxin-Induced Neurotoxicity. BMC Pharmacol. Toxicol. 2017, 18, 42.

Rabindran, S.K., He, H., Singh, M., Brown, E., Collins, K.I., Annable, A., Greenberger, L.M., 1998. Reversal of a Novel Multidrug Resistance Mechanism in Human Colon Carcinoma Cells by Fumitremorgin C. Cancer Res 58, 5850–5858.

Rannou, C.; Laroque, D.; Renault, E.; Prost, C.; Sérot, T. Mitigation Strategies of Acrylamide, Furans, Heterocyclic Amines and Browning during the Maillard Reaction in Foods. Food Res. Int. 2016, 90, 154–176.

RASFF. The Rapid Alert System for Food and Feed- 2020 Annual Report. European Commission 2020. 2020. Available online: https://ec.europa.eu/food/system/files/2021-08/rasff_pub_annual-report_2020.pdf (accessed on 8 January 2024).

Riahi, I., Pérez-Vendrell, A.M., Ramos, A.J., Brufau, J., Esteve-Garcia, E., Schulthess, J., Marquis, V., 2021. Biomarkers of Deoxynivalenol Toxicity in Chickens with Special Emphasis on Metabolic and Welfare Parameters. Toxins 2021, Vol. 13, Page 217 13, 217. https://doi.org/10.3390/TOXINS13030217

Richard, J.L., Bacchetti, P., Arp, L.H., 1981. Moldy walnut toxicosis in a dog, caused by the mycotoxin, penitrem A. Mycopathologia 76, 55–58. https://doi.org/10.1007/BF00761899/METRICS

Rifai, L.; Saleh, F.A. A Review on Acrylamide in Food: Occurrence, Toxicity, and Mitigation Strategies. Int. J. Toxicol. 2020, 39, 93–102.

Riss, T.; Niles, A.; Moravec, R.; Karassina, N.; Vidugiriene, J. Cytotoxicity Assays: In Vitro Methods to Measure Dead Cells. In Assay Guidance Manual; Eli Lilly & Company and the National Center for Advancing Translational Sciences: Bethesda, MD, USA, 2019.

Rundberget, T., Skaar, I., Flåøyen, A., 2004. The presence of Penicillium and Penicillium mycotoxins in food wastes. Int J Food Microbiol 90, 181–188. https://doi.org/10.1016/S0168-1605(03)00291-5

Sebastià, A.; Pallarés, N.; Bridgeman, L.; Juan-García, A.; Castagnini, J.M.; Ferrer, E.; Barba, F.J.; Berrada, H. A Critical Review of Acrylamide Green Extraction and Determination in Food Matrices: Current Insights and Future Perspectives. TrAC Trends Anal. Chem. 2023, 167, 117267.

Sharma, R.; Gettings, S.M.; Hazell, G.; Bourbia, N. In Vitro Study of Ochratoxin A in the Expression of Genes Associated with Neuron Survival and Viability. Toxicology 2023, 483, 153376.

Shreeve, B.J., Patterson, D.S.P., Roberts, B.A., MacDonald, S.M., 1983. Tremorgenic fungal toxins. Vet Res Commun 7, 155–160. https://doi.org/10.1007/BF02228611/METRICS

Singh, J.; Kaur, G. Transcriptional Regulation of Polysialylated Neural Cell Adhesion Molecule Expression by NMDA Receptor Activation in Retinoic Acid-Differentiated SH-SY5Y Neuroblastoma Cultures. Brain Res. 2007, 1154, 8–21.

Soares, C.M.D.; Alves, R.C.; Oliveira, M.B.P.P. Factors Affecting Acrylamide Levels in Coffee Beverages. Coffee Health Dis. Prev. 2015, 32, 217–224.

Song, G.; Liu, Z.; Wang, L.; Shi, R.; Chu, C.; Xiang, M.; Tian, Q.; Liu, X. Protective Effects of Lipoic Acid against Acrylamide- Induced Neurotoxicity: Involvement of Mitochondrial Energy Metabolism and Autophagy. Food Funct. 2017, 8, 4657–4667.

Stockmann-Juvala, H.; Mikkola, J.; Naarala, J.; Loikkanen, J.; Elovaara, E.; Savolainen, K. Oxidative Stress Induced by Fumonisin B1 in Continuous Human and Rodent Neural Cell Cultures. Free Radic. Res. 2009, 38, 933–942.

Stockmann-Juvala, H.; Naarala, J.; Loikkanen, J.; Vähäkangas, K.; Savolainen, K. Fumonisin B1-Induced Apoptosis in Neuroblastoma, Glioblastoma and Hypothalamic Cell Lines. Toxicology 2006, 225, 234–241.

Sumizawa, T.; Igisu, H. Apoptosis Induced by Acrylamide in SH-SY5Y Cells. Arch. Toxicol. 2007, 81, 279–282.

Sumizawa, T.; Igisu, H. Release of Heat Shock Proteins from Human Neuroblastoma Cells Exposed to Acrylamide. J. Toxicol. Sci. 2008, 33, 117–122.

Sun, Y.; Jiang, J.; Mu, P.; Lin, R.; Wen, J.; Deng, Y. Toxicokinetics and metabolism of deoxynivalenol in animals and humans. Arch. Toxicol. 2022, 96, 2639–2654.

Taeymans, D.; Wood, J.; Ashby, P.; Blank, I.; Studer, A.; Stadler, R.H.; Gondé, P.; Eijck, P.; Lalljie, S.; Lingnert, H.; et al. A Review of Acrylamide: An Industry Perspective on Research, Analysis, Formation, and Control. Crit. Rev. Food Sci. Nutr. 2004, 44, 323–347.

Tareke, E.; Rydberg, P.; Karlsson, P.; Eriksson, S.; Törnqvist, M. Analysis of Acrylamide, a Carcinogen Formed in Heated Foodstuffs. J. Agric. Food Chem. 2002, 50, 4998–5006.

Tepe, Y.; Çebi, A. Acrylamide in EnvironmentalWater: A Review on Sources, Exposure, and Public Health Risks. Expo. Health 2019, 11, 3–12.

Tola, M.; Kebede, B. Occurrence, Importance and Control of Mycotoxins: A Review. Cogent Food Agric. 2016, 2, 1191103.

Venkataramana, M.; Chandra Nayaka, S.; Anand, T.; Rajesh, R.; Aiyaz, M.; Divakara, S.T.; Murali, H.S.; Prakash, H.S.; Lakshmana Rao, P.V. Zearalenone Induced Toxicity in SHSY-5Y Cells: The Role of Oxidative Stress Evidenced by N-Acetyl Cysteine. Food Chem. Toxicol. 2014, 65, 335–342.

Walter, S.L., 2002. Acute penitrem A and roquefortine poisoning in a dog. Canadian Veterinary Journal 43, 372–374.
Walter, S.L., 2002. Acute penitrem A and roquefortine poisoning in a dog. Canadian Veterinary Journal 43, 372–374.

Wang, L.; Huang, Q.; Wu, J.; Wu, W.; Jiang, J.; Yan, H.; Huang, J.; Sun, Y.; Deng, Y. The metabolism and biotransformation of AFB1: Key enzymes and pathways. Biochem. Pharmacol. 2022, 199, 115005.

WILSON, B., 1971. MISCELLANEOUS PENICILLIUM TOXINS. MISCELLANEOUS PENICILLIUM TOXINS.
Wilson, B., 1971. Miscellaneous penicillium toxins. Miscellaneous penicillium toxins.

Wu, L., Wang, B., 2016. Transformation of deoxynivalenol and its acetylated derivatives in Chinese steamed bread making, as affected by pH, yeast, and steaming time. Food Chem 202, 149–155. https://doi.org/10.1016/J.FOODCHEM.2016.01.124

Xiang, J.; Liu, F.; Wang, B.; Chen, L.; Liu, W.; Tan, S. A Literature Review on Maillard Reaction Based on Milk Proteins and Carbohydrates in Food and Pharmaceutical Products: Advantages, Disadvantages, and Avoidance Strategies. Foods 2021, 10, 1998.

Xie, H.R.; Hu, L.S.; Li, G.Y. SH-SY5Y Human Neuroblastoma Cell Line: In Vitro Cell Model of Dopaminergic Neurons in Parkinson's Disease. Chin. Med. J. 2010, 123, 1086–1092.

Yan, D.; Pan, X.; Yao, J.; Wang, D.; Wu, X.; Chen, X.; Shi, N.; Yan, H. MAPKs and NF-KB-Mediated Acrylamide-Induced Neuropathy in Rat Striatum and Human Neuroblastoma Cells SY5Y. J. Cell Biochem. 2019, 120, 3898–3910.

Yan, D.;Wang, N.; Yao, J.;Wu, X.; Yuan, J.; Yan, H. Curcumin Attenuates the PERK-EIF2 Signaling to Relieve Acrylamide-Induced Neurotoxicity in SH SY5Y Neuroblastoma Cells. Neurochem. Res. 2022, 47, 1037–1048.

Yoon, S.; Cong, W.T.; Bang, Y.; Lee, S.N.; Yoon, C.S.; Kwack, S.J.; Kang, T.S.; Lee, K.Y.; Choi, J.K.; Choi, H.J. Proteome Response to Ochratoxin A-Induced Apoptotic Cell Death in Mouse Hippocampal HT22 Cells. Neurotoxicology 2009, 30, 666–676.

Young, K.L., Villar, D., Carson, T.L., Imerman, P.M., Moore, R.A., Bottoff, M.R., 2003. Tremorgenic mycotoxin intoxication with penitrem A and roquefortine in two dogs. J Am Vet Med Assoc 222, 52–53. https://doi.org/10.2460/JAVMA.2003.222.52

Yu, C.P.; Pan, Y.L.; Wang, X.L.; Xin, R.; Li, H.Q.; Lei, Y.T.; Zhao, F.F.; Zhang, D.; Zhou, X.R.; Ma, W.W.; et al. Stimulating the Expression of Sphingosine Kinase 1 (SphK1) Is Beneficial to Reduce Acrylamide-Induced Nerve Cell Damage. Ecotoxicol. Environ. Saf. 2022, 237, 113511.

Zain, M.E. Impact of Mycotoxins on Humans and Animals. J. Saudi Chem. Soc. 2011, 15, 129–144.

Zhang, X.; Boesch-Saadatmandi, C.; Lou, Y.;Wolffram, S.; Huebbe, P.; Rimbach, G. Ochratoxin A induces apoptosis in neuronal cells. Genes Nutr. 2009, 4, 41.

2.OBJECTIVES

2. Objetivos

2. OBJETIVOS

El objetivo de la presente Tesis Doctoral es estudiar los efectos toxicológicos tanto individuales como combinados de acrilamida (AA), penitrem A (PEN A), fumitremorgin C (FTC) y 3-acetildeoxinivalenol (3-ADON) mediante ensayos *in vitro* en células SH-SY5Y. Para llevar a cabo este objetivo se proponen los siguientes objetivos específicos:

- Revisar y analizar críticamente la evidencia científica disponible sobre los efectos tóxicos de la acrilamida y citadas micotoxinas en la línea celular de neuroblastoma humano SH-SY5Y, con especial atención en los mecanismos de neurotoxicidad, estrés oxidativo, citotoxicidad y posibles interacciones en exposiciones combinadas.

- Determinar la naturaleza de los efectos combinados —ya sean sinérgicos, aditivos o antagónicos de AA, FTC y PEN A en células de neuroblastoma humano SH-SY5Y; mediante la integración del análisis de isobologramas con la evaluación de la citotoxicidad. Así mismo se evaluará cómo la coexposición a estos contaminantes alimentarios afecta puntos clave de regulación del ciclo celular, mediante el estudio por citometría de flujo.

- Conocer el desequilibrio redox provocado por AA, PEN A y 3-ADON y la cuantificación de biomarcadores como la producción de especies reactivas de oxígeno (ROS), los niveles de lipoperoxidación (LPO) y la actividad de enzimas antioxidantes en células humanas de neuroblastoma SH-SY5Y expuestas a los contaminantes del procesado alimentario e de origen natural, tanto de manera individual como combinada. De esta forma se pretende conocer información sobre los mecanismos subyacentes de la neurotoxicidad y los posibles efectos acumulativos o interactivos de contaminantes alimentarios naturales y del procesado.

- Evaluar los efectos genotóxicos de la exposición a AA, PEN A y 3-ADON de manera individual y combinado en células humanas de neuroblastoma SH-SY5Y, mediante el ensayo de micronúcleos (MN) y el test de Ames. Esto permitirá detectar alteraciones cromosómicas a nivel celular, así como obtener información sobre la actividad mutagénica a nivel genético.

2. OBJECTIVES

The objective of the present Doctoral Thesis is to study the individual and combined toxicological effects of acrylamide (AA), penitrem A (PEN A), fumitremorgin C (FTC), and 3-acetyldeoxynivalenol (3-ADON) through in vitro assays using SH-SY5Y cells. To achieve this objective, the following specific aims are proposed:

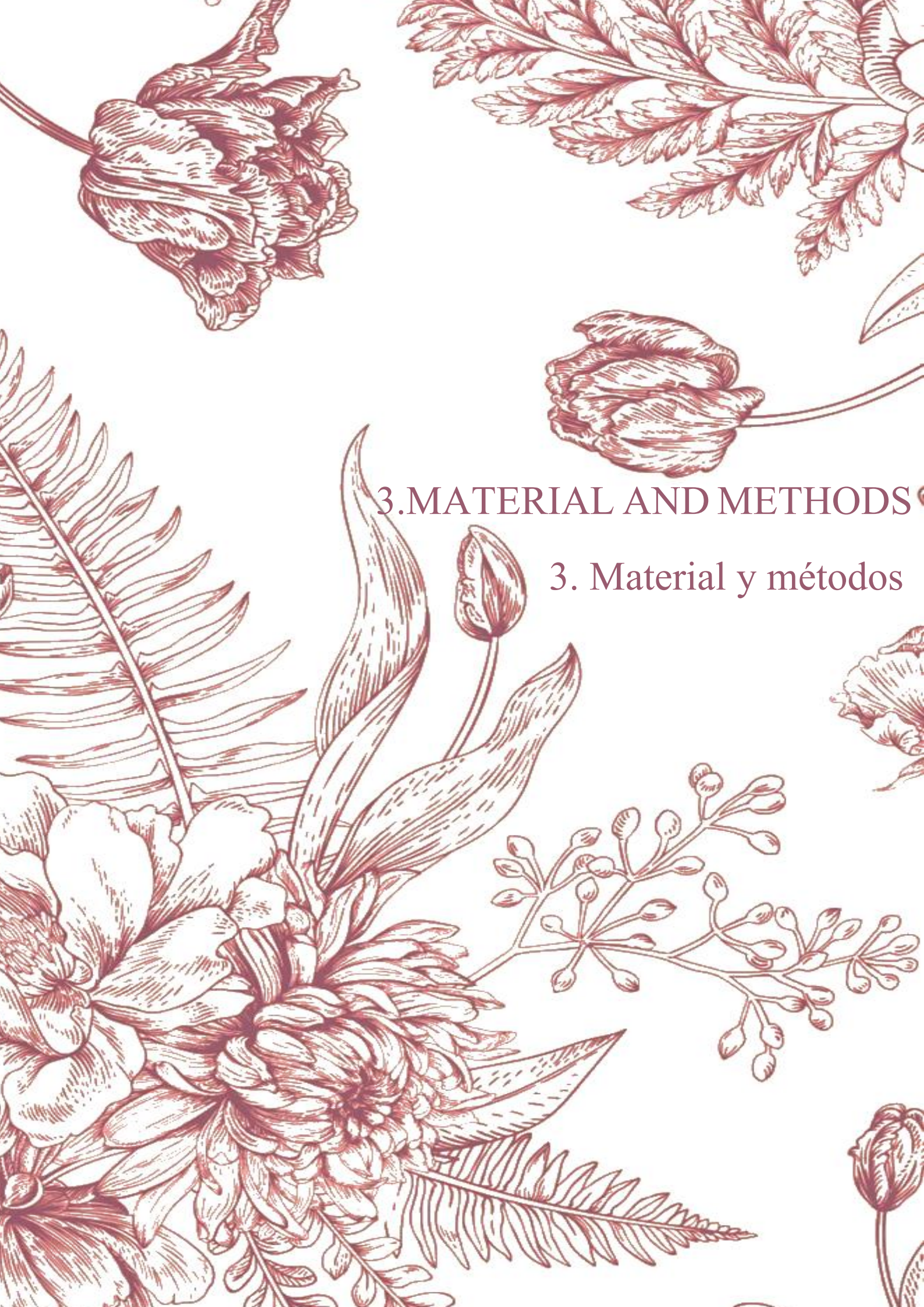
- To critically review and analyze the available scientific evidence on the toxic effects of acrylamide and various mycotoxins in the human neuroblastoma cell line SH-SY5Y, with particular emphasis on mechanisms of neurotoxicity, oxidative stress, cytotoxicity, and possible interactions in combined exposures.

- To determine the nature of the combined effects—whether synergistic, additive, or antagonistic—of AA, FTC, and PEN A in SH-SY5Y neuroblastoma cells by integrating isobologram analysis with cytotoxicity assessment. Additionally, the impact of co-exposure to these food contaminants on key cell cycle regulation points will be evaluated by flow cytometry.

- To assess the redox imbalance induced by AA, PEN A and 3-ADON through the quantification of biomarkers such as reactive oxygen species (ROS), lipid peroxidation (LPO) levels, and the activity of antioxidant enzymes in SH-SY5Y human neuroblastoma cells exposed to food processing and naturally occurring contaminants, both individually and in combination. This aim seeks to elucidate the underlying mechanisms of neurotoxicity and the potential cumulative or interactive effects of natural and processed food contaminants.

- To evaluate the genotoxic effects of individual and combined exposure to AA, PEN A, and 3-ADON in SH-SY5Y human neuroblastoma cells using the micronucleus (MN) assay and the Ames test. This will allow for the detection of

chromosomal alterations at the cellular level and provide insights into mutagenic activity at the genetic level.



3.MATERIAL AND METHODS

3. Material y métodos

3. MATERIALS AND METHODS

3.1. Reagents

The cell culture components and reagent-grade chemicals used in the experiments included Dulbecco's Modified Eagle's Medium-F12 (DMEM/F-12), Minimum Eagle's Medium (MEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), penicillin, streptomycin, trypsin-EDTA, and 100× non-essential amino acids. These were supplied by Thermo Fisher (Gibco™, Paisley, UK), Sigma-Aldrich (St. Louis, MO, USA), and Camber Bio Science (Verviers, Belgium). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific Co. (Fisher BioReagents™, Geel, Belgium; Madrid, Spain), and deionized water (<18 MΩ cm resistivity) was produced in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Bradford, MA, USA). Additional chemicals included thiobarbituric acid (TBA), di-ter-butyl-methylphenol (BHT), 2′,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA), β-nicotinamide adenine dinucleotide phosphate (β-NADPH), sodium azide (NaN3), t-octylphenoxypolyethoxyethanol (Triton-X 100), hydrogen peroxide (H2O2), and methanol (MeOH), all from Sigma-Aldrich. The standards of acrylamide (AA, MW: 71.08 g/mol, CAS: 79-06-1), Penitrem A (PEN A, MW: 634.2 g/mol, CAS: 12627-35-9), and 3-acetyldeoxynivalenol (3-ADON, MW: 338 g/mol, CAS: 50722-38-8) were also obtained from Sigma-Aldrich. Mycotoxin stock solutions were prepared in DMSO or H2O, as appropriate, and maintained at −20 °C in the dark. Additional reagents included mitomycin C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for MTT assays, and Ames test positive controls [2-nitrofluorene (2-NF), sodium azide (SA), 4-nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2-AA)]. The Litron In Vitro Microflow Kit for the micronucleus assay by flow cytometry was purchased from Litron Laboratories (Rochester, NY, USA).

3.2. Cell culture

SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F-12) or Dulbecco's Modified

Eagle's Medium (DMEM, Sigma–Aldrich), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma–Aldrich). Cells were maintained as monolayers in 150 cm^2 cell culture flasks with filter screw caps (TPP, Trasadingen, Switzerland) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Sub-cultivation was performed once or twice a week by trypsinization (Trypsin-EDTA, Sigma–Aldrich) and resuspension in complete medium in a 1:3 split ratio. The culture medium was refreshed every 2–3 days, and final tested concentrations were obtained by adding compound solutions, ensuring that the final DMSO or methanol concentration did not exceed 1% (v/v). Each experiment included appropriate controls containing the same amount of solvents.

3.3. Cell treatment

SH-SY5Y cells were cultured into 96-well tissue-culture plates at 2×10^6 cells/ml. After 24 h, the culture medium was replaced, and cells were treated during 24 h, 48 h and 72 h to 200 µl of fresh medium containing different concentrations of i) AA from 9.8 to 5000 µM; or ii) FTC from 0.0097 to 5 µM; or iii) PEN A from 0.05 to 15 µM the maximum doses for the three compounds were chosen taken under consideration the doses that have been previously established in literature (Berntsen et al., 2017b; Kerr, 2009; Okuno et al., 2006; Sridhar K Rabindran et al., 1998; Sabater-Vilar et al., 2003; Song et al., 2017; Yan et al., 2019). Also, the mycotoxins were assayed in combination in the following mixtures: AA + FTC (9.77 – 2500 µM) + (0.027 – 5 µM), AA + PEN A (9.77 – 2500 µM) + (0.10 – 20 µM), FTC + PEN A (0.10 – 20 µM) + (0.027 – 5 µM) and AA + FTC + PEN A (9.77–0.027 – 0.10 µM) + (2500–5 – 20 µM) at 24h, 48h and 72h. (Table 13). The dilution ratios of the concentrations for the binary combinations were 1000:1 for AA + FTC, 250:1 for AA + PEN A, 4:1 for PEN A + FTC and 1000:250:1 for the tertiary combination (AA + FTC + PEN A).

Table 13. Concentration range (µM) of mycotoxins studied individually and in combinations.

Compounds tasted	Concentration ranges (µM)
AA	19.6 – 5000
FTC	0.027 – 5
PEN A	0.10 – 20
AA + FTC	(9.77 - 2500) + (0.027 - 5)
AA +PEN A	(9.77 - 2500) + (0.10 - 20)
FTC + PEN A	(0.027 – 5) + (0.10 - 20)
AA + FTC + PENA	(9.77 - 2500) + (0.027 - 5) + (0.10 - 20)

3.4. Oxidative stress

SH-SY5Y cells were cultured into 96-well plates at 2×10^6 cells/plate. After 24 h, the culture medium was replaced, and cells were treated during 24 h to 200 µl of fresh medium containing different concentrations of i) AA at 1250 µM, 2500 µM and 5000 µM; or ii) PEN A at 5 µM, 10 µM and 20 µM or iii) 3-ADON at 1 µM, 2 µM and 4 µM. The maximum doses for the three compounds were chosen taken under consideration the doses that have been previously established in literature and the cytotoxic study carried out by Bridgeman et al., (2023). Also, the mycotoxins were assayed in combination in the following mixtures: AA + PEN A at [1250 + 5] µM, [2500 + 10] µM, [5000 + 20] µM , AA + 3-ADON at [1250 + 1] µM, [2500 + 2] µM, [5000 + 4] µM; and PEN A + 3-ADON at [5 + 1] µM, [10 + 2] µM, [20 + 4] µM, and AA + PEN A + 3-ADON at [1250 + 5 + 1] µM[5000 + 20 + 4] µM at 24h. The dilution ratios of the concentrations for the binary combinations were 250:1, 1250:1 and 5:1, respectively, and 1250:250:1 for the tertiary combination (Table 14).

Table 14. *Concentrations and ratios of AA, PEN A y 3-ADON tested individually and in combination.*

Compound tested	Ratio	Concentrations (µM)
AA		1250, 2500, and 5000
PEN A		5, 10, and 20
3-ADON		1, 2, and 4
AA + PEN A	250:1	[1250 + 5], [2500 + 10], and [5000 + 20],
AA + 3-ADON	625:1	[1250 + 1], [2500 + 2], and [5000 + 4]
PEN A + 3-ADON	5:1	[2.5 + 1], [5 + 2], and [10 + 4]
AA + PEN A + 3-ADON	625:250:1	[1250 + 5 + 1], [2500 + 10 + 2], and [5000 + 20 + 4]

3.5. Mycotoxin exposure for genotoxicity and mutagenicity

In this study, concentration of food contaminants and exposure time were two important factors to consider, high concentrations of exposure were selected to study acute toxicity because they allow the identification of threshold and maximum toxic effects, provide mechanistic insights, and simulate worst-case scenarios, which are essential for understanding the risks of compounds like AA, PEN A, and 3-ADON. Furthermore, the concentrations were selected based on the cytotoxicity results previously reported for the individual and combined exposures of AA PEN A and 3-ADON in SH-SY5Y cells ensuring that are both safe and biologically relevant for the assays (above IC_{50}) (Bridgeman et al., 2023). Individual treatment was assayed at a concentration range of 625, 1250, and 2500 µM for AA, at 2.5, 5, and 10 µM for PEN A, and at 1, 2, and 4 µM for 3-ADON. For combination mixtures, individual treatment data were crucial for selecting starting concentration assuring that the highest concentration did not exceed the IC > 55 ± 5%. Nevertheless, parallel assays of individual and combinations were performed for exact evaluation of combinatory effects. Concentration for combinations of both mycotoxins at a dilution ratio of 250:1 [AA + PEN A], 625:1 [AA + 3-ADON], 5:1 [PEN A + 3-ADON], and 625:250:1 for the tertiary combination (Table 15).

***Table 15**. Concentrations and ratios of AA, PEN A y 3-ADON tested individually and in combination.*

Compound tested	Ratio	Concentrations (µM)
AA		625, 1250, and 2500
PEN A		2.5, 5, and 10
3-ADON		1, 2, and 4
AA + PEN A	250:1	[625 + 2.5], [1250 + 5], and [2500 + 10],
AA + 3-ADON	625:1	[625 + 1], [1250 + 2], and [2500 + 4]
PEN A + 3-ADON	5:1	[2.5 + 1], [5 + 2], and [10 + 4]
AA + PEN A + 3-ADON	625:250:1	[625 + 2.5 + 1], [1250 + 5 + 2], and [2500 + 10 + 4]

The concentrations tested in the Ames test (using different bacterial strains) were the same as those selected for SH-SY5Y cells, as described above. Given the genotoxicity results observed in both individual and combined treatments, we applied these same conditions to assess mutagenicity of these concentrations in particular.

3.6. MTT assay

The MTT assay was employed to assess cell viability. This assay relies on the metabolic reduction of a yellow, soluble tetrazolium salt by metabolically active cells. The reduction is facilitated by the mitochondrial enzyme succinate dehydrogenase, converting the tetrazolium salt into insoluble purple formazan crystals. The MTT assay has been extensively used to study the cytotoxicity of mycotoxins as described in previous works of our research group (Bridgeman et al., 2023; Juan-García et al., 2016; Penalva-Olcina et al., 2022). In brief, after exposure to the test compounds, the medium was removed, and 200 µL of fresh medium containing 50 µL of MTT solution (5 mg/ml; MTT powder dissolved in PBS) was added to each well. The plates were then kept away from light and incubated at 37°C for 4 hours. At the end of the experiment, the MTT-containing medium was removed from the plates, and 200 µL of DMSO along with 25 µL of Glycine-Sorensen's solution was added to each well. Absorbance was then measured at 570 nm using an ELISA Multiskan EX (Thermo Scientific, MA, USA). Cell viability was determined as a percentage compared to control cells treated with 1% H_2O for AA, 1% DMSO for FTC, PEN A and 3-ADON. Mean inhibition concentration

(IC_{50}) values were calculated from full dose–response curve. Three independent experiments were performed with eight replicates each.

3.7. Cell cycle analysis

Cell cycle analysis was performed by flow cytometry, using Vindelov's propidium iodide (PI) staining solution as described previously by Juan-García et al. (2013). The PI solution is a fluorescent DNA intercalating agent able to bind and label double-stranded nucleic acids, making it possible to achieve rapid and precise evaluation of cellular DNA content by flow cytometric analysis. The cell cycle is monitored by different key checkpoints or decision points on whether the cell should divide, delay division, or enter a resting stage. For this purpose, SH-SY5Y cells were seeded at 7×10^5 cells/well in six-well plates. After 90% of confluence, cells were treated with AA (2500, 1250 and 625 µM), FTC (5 µM), and PEN A (20, 10 and 5 µM) as an individual treatment. In the combinations assays the following mixtures were tested: AA + FTC, AA + PEN A, FTC + PEN A and AA + FTC + PEN A with concentrations ranged from 2505 to 626,25 µM, 2520 to 630 µM, and 25 to 10 µM (Table 13). Then, cells were trypsinized and removed after 3 min of incubation, then they were placed on ice for 30 min with of fresh medium containing 0.5 mL Vindelov's PI staining solution prepared as follows: 40 µg/mL RNAase, 0.1 % Triton X-100, 10 mM Tris, 10 mM NaCl and 50 µg/mL of PI in PBS. Twenty thousand cells for each sample were analyzed using BD LSRFortessa (BD Biosciences) flow cytometry. The experiments were carried out in duplicate, and the results were expressed as the mean ± SEM of different independent experiments.

3.8. Experimental design and combination index

In order to determine the type of interaction (synergism, additive effect, and antagonism) that occurred when AA, FTC, and PEN A were in combination, the isobologram analysis (Chou–Talalay model) was used. This model allows characterizing the interactions induced by combinations of AA, FTC, and PEN A in different cell lines and with different compounds, but it does not allow the elucidation of the mechanisms by which these types of interaction are produced (Table 13). The

median effect/ combination index (CI) isobologram equation by Chou (2006) and Chou and Talalay (1984b) permitted analysing 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: $\frac{fa}{fu} = (\frac{D}{Dm})^m$ Parameters such as Dm (median effect dose), f a (fraction affected by concentration), and m (coefficient signifying the shape of the dose–effect relationship) are relevant in the equation. Therefore, the method considers both potency (Dm) and shape (m) parameters. Chou and Talalay (1984b) introduced the term CI. CI values < 1, =1, and >1 indicate synergism, additive effects, and antagonism of the combination, respectively. CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007) was used to study the types of interactions assessed by the isobologram analysis. The IC_{25}, IC_{50}, IC_{75}, and IC_{90} are the doses required to produce toxicity at 25 %, 50 %, 75 %, and 90 %, respectively.

3.9. Intracellular ROS generation

Early intracellular ROS production was tracked in SH-SY5Y cells by adding H_2-DCFDA. This compound is absorbed by cells and deacetylated by intracellular esterases. The resulting non-fluorescent 2′, 7′-dichlorodihydrofluorescein (H_2-DCF) becomes highly fluorescent dichlorofluorescein (DCF) upon oxidation by ROS. The generation of ROS was monitored according to Juan-García et al. (2020). Briefly, 3×10^4 cells/well were seeded in a 96-well black culture microplate. Once the cells reach 90 % confluence, the culture medium was replaced, and cells were loaded with 20 µM H_2-DCFDA in fresh medium for 20 min. Subsequently H_2-DCFDA was removed and 200 µl/well of fresh medium, 1 % DMSO (control) or medium with AA, PEN A and 3-ADON individually or in combination was added. The selection of the three different concentrations assayed is correlated with those found in food and were selected according to the previous cytotoxic assays carried out. Increases in fluorescence were measured on a multimode microplate reader (Biotek Synergy H1; Agilent, GA, USA) at intervals up to 120 min at excitation/emission wavelengths of 485/535 nm. Results are expressed as increase in fluorescence respect to solvent control.

Determinations were performed in two independent experiments with 24 replicates each. H_2O_2 was used as a positive control (Table 14).

3.10. Lipid peroxidation assay

Lipid peroxidation (LPO) assay was carried out by determining the formation of reactive thiobarbituric acid reactive substances (TBARS), that allows to determine the production of a red adduct between TBA and malondialdehyde (MDA), which is a biomarker used to prove that the LPO process has occurred. Briefly, 7×10^5 cells/well were seeded in six-well plates. Once the cells reached 90 % confluence, cells were exposed to AA, PEN A and 3-ADON individually or in combination for 24 h. Then, the medium was removed, and cells were washed with PBS, homogenized in 150 mM sodium phosphate buffer (NaH_2PO_4) pH 7.4 and lysate with the Ultra-Turrax T8 IKA®-WERKE. Immediately, cells were boiled at 100 °C in water bath for 20 min under acid condition in the presence of 0.5 % TBA, 1.5 mM DFA and 3.75 % BHT. After that, the samples were placed on ice for 5 min and centrifuged at 5000 rpm for 15 min. The absorbance was measured at 532 nm. Two independent experiments were conducted. Results were expressed as ng of MDA/mg of protein measured. The protein content was determined by the Bio-Rad DC Protein Assay (catalog number 500–0116). Briefly, we added 5µL of sample, 25µL of reagent A and 200 µL of reagent B in each well of a 96 well plate, and it was shacked 20 min. Finaly, the protein concentration (µg/ml) was measured at 690 nm (Table 14).

3.11. GSH and GSSG determination

In short, 7×10^5 cells per well were seeded in six-well plates. After 24 hours, the culture medium was replaced with fresh medium containing varying concentrations of AA (1250 µM, 2500 µM, and 5000 µM), PEN A (5 µM, 10 µM, and 20 µM), and 3-ADON (1 µM, 2 µM, and 4 µM), both individually and in combination, and incubated for another 24 hours (Table 14). Following this, the medium was removed, and the cells were washed with PBS and homogenized in 0.25 ml of 20 mM Tris and 0.1 % Triton. For GSH determination, 10 µL of each homogenized cell sample was added to a 96-

well black tissue culture plate with 200 µL of GSH buffer (pH 8.0) and 10 µL of OPT solution, mixed, and incubated in the dark at room temperature for 15 minutes. For GSSG determination, 25 µL of each homogenized cell sample and 25 µL of NEM (N-ethylmaleimide, 0.005 g/mL in deionized water) were mixed in a 1.5 ml Eppendorf tube and incubated at room temperature for 20 minutes. Then, 50 µL of 0.1 N NaOH was added to adjust the pH for the GSSG assay. A 10 µL portion of this mixture was placed in a 96-well black tissue culture plate with 200 µL of 1 N NaOH and 10 µL of OPT (O-phthalaldehyde, 0.001 g/mL in MeOH) solution, mixed, and incubated in the dark at room temperature for 15 minutes.

GSH and GSSG concentrations (prepared in the plates described above) were measured using a multimode microplate reader (Biotek Synergy H1; Agilent, GA, USA) with excitation and emission wavelengths of 345 nm and 424 nm, respectively. The levels of GSH and GSSG were expressed as µg/mg of protein. The protein content was determined by the Bio-Rad DC Protein Assay (catalogue number 500–0116). Briefly, we added 5µL of sample, 25µL of reagent A and 200 µL of reagent B in each well of a 96 well plate, and it was shacked 20 min. Finaly, the protein concentration (µg/ml) was measured at 690 nm. These determinations were conducted in two independent experiments, each with 4 replicates.

3.12. Micronucleus (MN) assay by flow cytometry

Micronucleus assay was carried out using the Litron In Vitro Microflow kit (Litron Laboratories, Rochester, NY). The assay was performed according to the manufacturer´s instructions based on previous reports (Bryce et al., 2008). Briefly, 2 × 10^6 cells/well were seeded in 24-well plates and exposed to 625, 1250, and 2500 µM of AA, to 2.5, 5, and 10 µM of PEN A, and to 1, 2, and 4 µM of 3-ADON for 24 h, SH-SY5Y cells were cultured in fresh medium for an additional period until completing 48 h, which is equal to 1.5–2 doubling times for this cell line (Table 15). On the day of the experiment, cells were stained with the nucleic acid dye A (EMA) solution and placed on the ice near a light source for 30 min, to induce its photoactivation. The EMA

fluorochrome dye is a reagent that crosses the compromised outer membrane of apoptotic and necrotic cells and binds to DNA through photoactivation. Then, cells were washed, lysed with the Litron Lysis kit solution, and preserved from light for 60 min. During the lysis step, cytoplasmic membranes were digested to liberate nuclei and MN. Finally, a lysis solution containing SYTOX fluorochrome, which labels all chromatin, was added and cells were incubated for 30 min at room temperature in darkness. The differential staining allows the distinction between healthy chromatin and dead/dying cells. Analysis was performed by using a BD FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry, following the instructions and templates provided by the Litron In Vitro Microflow Kit manual and as described by (Bryce et al., 2008). Following the OECD guideline 487 Mitomicin C (1.5 µM) was used as a positive control coinciding with reported literature (Fernández-Bertólez et al., 2021). Three independent experiments were performed. The results were expressed as a percentage of MN per 20,000 gated nuclei events per sample as indicated in OECD TG 487 for MN evaluation through flow cytometry.

3.13. Ames test

Due to the small amounts of mycotoxins the Miniaturised Ames test was performed strictly with the same protocol as the Ames test (preparation of cells, treatment) except that all reagent volumes were divided by five as reported by Flamand et al. (2001). With or without metabolic activation, four histidine-dependent auxotrophic mutants of *S. typhimurium* strains, TA 98, TA 100, TA 1535, TA 1537 were used with the 6-well plate integration approach previously described in literature (Al-Ayoubi et al., 2023; Burke et al., 1996; Flamand et al., 2001; Sanz-Serrano et al., 2021). As recommended by Brusick et al. (1980), a fifth strain was used: a tryptophan-dependent auxotrophic mutant *Escherichia coli (E. Coli WP2*) (Table 16). The test strains were cultured in the liquid broth medium for 10 h at 37 °C under agitation. After incubation, 80 µl of the bacterial culture were mixed in 1.6 ml of top agar with 80 µl of test compound dilution and, for the activated tests, with 450 µl of S9 mix. After mixing 530 µl/well of the resulting mix were poured into six-well plates (35-mm dishes) containing 5 ml of

minimal media. As soon as the soft agar solidified, the six-well plates were incubated at 37°C for 48 h. For the experiments with *S. typhimurium*, the top agar was supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL agar, and mutations to histidine independence were scored on minimal glucose agar plates. For experiments with *E. coli* WP2 strain, mutations to tryptophan independence were scored on minimal glucose agar plates supplemented with 10 mL of 0.5 mM tryptophan per 100 mL agar. After 48 h, the number of revertant colonies was counted. All experiments were carried out in triplicate using 3 concentrations of each compound. The average number of revertant colonies and the standard deviation (SD) of three replicates for samples, positive and negative controls were calculated. According to the historical values in the laboratory, a compound tested with the Ames test was considered mutagenic if the number of His^+ revertant colonies was at least twice or thrice the value of the corresponding solvent control depending the strain; for the strain in our study as follows: induction factor >2 for *TA98, TA100 and E.coli WP2* and >3 for *TA 1537 and TA1535*. A dose–effect relationship is an additional indication for the mutagenic potency of a compound. A possible mutagenic potential is assumed if the quotient ranges between 1.7 and 1.9 in combination with an observed dose–effect relationship. No mutagenic potential is assumed if all quotients range between 1.0 (or lower) and 1.6. The latter conclusions may be strengthened by the lack of observation of a dose–effect relationship. Otherwise, a negative result was defined.

***Table 16**. Ames test strains explanation.*

Strains	**Affected genes**	**Additional Deployments**		**Plasmid**	**Type of mutation detected**
		Repair	LPS		
TA98	hisD3052	uvrB	frg	pKM101	Frameshift
TA100	hisG46	uvrB	frg	pKM101	Base Substitution
TA1535	hisG46	uvrB	frg	-	Base Substitution
TA1537	hisC3076	uvrB	frg	-	Frameshift
WP2 uvrA (pKM101)	trpE	uvrA	Not applicable	pKM101	Base Substitution

3.14. Statistical analysis

Statistical analysis was conducted using both IBM SPSS Statistics version 23.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 7 (GraphPad Software, California, USA). Data were expressed as mean ± SD or SEM from independent experiments. Statistical comparisons were performed using Student's t-test for paired samples, while differences between groups were analyzed using one-way ANOVA followed by the Tukey HSD post hoc test for multiple comparisons. A p-value of ≤ 0.05 was considered statistically significant.

4. RESULTS

4. Resultados

CHAPTER I: 4.1. Individual and combined effect of acrylamide, fumitremorgin C and penitrem A on human neuroblastoma SH-SY5Y cells

4.1.1. Introduction

AA, FTC, and PEN A are all chemical compounds that can be found in certain foods and have been associated with potential health risks (Fig. 1). AA is formed when certain foods are cooked at high temperatures, particularly in processes such as frying, baking, or roasting (Sarion et al., 2021; Sebasti`a et al., 2023). Foods that are high in starch, such as potatoes, bread, and coffee, are particularly prone to AA formation. AA has been linked to an increased risk of cancer in animal studies, although its effects on humans are still being studied (Klaunig, 2008a; IARC). AA is classified as probable carcinogen (2A) substance by the International Agency for Research on Cancer (IARC) (IARC, 1994). Exposure to AA has been linked to several neurological effects, including peripheral neuropathy, ataxia, and impaired cognitive function (Spencer and Schaumburg, 1974). Studies in animals have shown that AA can damage nerve cells and disrupt the balance of neurotransmitters in the brain, leading to various neurological symptoms (Allam et al., 2011; Klaunig, 2008b; Lapin et al., 1984). One of the mechanisms through which AA causes neurotoxicity is by forming adducts with proteins in nerve cells, which can alter their function and structure. AA can also disrupt the production of ATP, which is essential for the proper functioning of nerve cells (Erkekoglu and Baydar, 2014).

Besides, it has been demonstrated that humans can be exposed to a large number of mycotoxins through contaminated foods and feeds (Liu et al., 2022; Zhao et al., 2021), that is why on this studied we not only focused our attention on chemical contaminants, but also in two scarcely studied mycotoxins that can contaminate one of the main sources of AA (Nikolic et al., 2022). FTC and PEN A, toxic mycotoxins that are produced by certain species of fungi such as Penicillium crustosum and Aspergillus fumigatus (Kalinina et al., 2017; Yuan et al., 2020) and can contaminate some cereals such as corn (Dorner et al., 1984). Specifically, FTC is often present in contaminated cereals, grains, and hay, while PEN A is commonly found in contaminated nuts, dried fruits, and grains (Richard et al., 1986; Tournas, 2008). Both mycotoxins have been associated with adverse health effects in animals and humans, including tremors,

seizures, and muscle spasms (Berntsen et al., 2017a). FTC, belonging to a class of diketopiperazines that are potent mycotoxins.

FTC caused tremors in cockerels at 25 mg/kg orally (Cole and Cox, 1981). Also, FTC, at a 5 μM concentration, significantly increased the toxicity of mitoxantrone (93-fold), doxorubicin (26-fold), topotecan (24-fold), and bisantrene (25-fold) (Sridhar K Rabindran et al., 1998). At 1 μM, FTC was also identified as a breast cancer resistance protein (BCRP/ABCG2) and cancer drug resistance demonstrated to increase the amounts of mitoxantrone, and doxorubicin retained by S1-M1-3.2 cells (Sridhar K Rabindran et al., 1998). In the same line, in these studies, FTC has shown to be a specific and selective inhibitor of the BCRP (Garimella et al., 2004).

On the other hand, PEN A can impair GABAergic amino acid neurotransmission and selectively antagonize high-conductance Ca+2-activated potassium (BK) channels. Dysfunctional neurotransmission and inhibition of neuronal BK channels are probably involved in the neurotoxic actions observed in both humans and animals (Berntsen et al., 2017a; Eriksen et al., 2010; Moldes-Anaya et al., 2011). Many intoxications with the mycotoxin PEN A in animals have been reported recurrently in the literature (Breton et al., 1998; Eriksen et al., 2010; Moldes-Anaya et al., 2009; Walter, 2002) and some cases have been reported in humans (Lewis et al., 2005). The incidence of PEN A-induced neuromycotoxicosis may be under-diagnosed, especially in humans, because of the lack of knowledge about PEN A's deleterious effects and the difficult access to appropriate analytical tools for detection and determination of this neurotoxin (Berntsen et al., 2017a). While AA, FTC, and PEN A are distinct chemical compounds with different properties and mechanisms of action, they are all examples of potential health risks to humans and animals that can co-occur in certain grains, that can be a potential source of AA (Dorner et al., 1984; Mesias et al., 2022). Therefore, studying the combination of contaminants provides a more realistic understanding of the potential health risks associated with the consumption of contaminated food. Also, the presence of multiple contaminants can result in synergistic, antagonist and additive effects and the toxicity of a contaminant can be increased when it interacts with other

contaminants, independently of the level present in food. The regulatory limits for individual contaminants may not take into account the potential additive or synergistic effects of multiple contaminants. Therefore, studying the combination of contaminants can help inform the development of more comprehensive regulatory guidelines for food safety. Overall, studying the impact of the combinations of food contaminants in toxicology is crucial for understanding the potential health risks associated with contaminated food and for developing effective strategies to mitigate these risks.

Therefore, studying the cytotoxic interaction between them and the cell cycle disruption of AA, FTC, and PEN A in SH-SY5Y cells is important for better understanding their mechanism of action and the potential risks they pose to human health. The objective of the present study was to investigate the cytotoxicological interactions between AA, FTC, and PEN A in human neuroblastoma SH-SY5Y cells. The effects of individual and combinations of two and three compounds were evaluated by isobologram analysis (Chou and Talalay, 1984a) to determine whether their interaction was synergistic, additive, or antagonistic, as well as to understand how food contaminants can act at the cellular level. Among that the cell cycle distribution was analyzed either in individual or in combined treatment.

4.1.2. Materials and methods

- Reagents (described in section 3.1.)

- Cell culture (described in section 3.2.)

- Cell treatment (described in section 3.3.)

- MTT assay (described in section 3.4.)

- Cell cycle analysis (described in section 3.5.)

- Experimental design and combination index (described in section 3.6.)

- Statistical analysis (described in section 3.14)

4.1.3. Results

4.1.3.1. *Cytotoxicity effects of individual exposure to AA, FTC, and PEN A*

The cytotoxicity effects of AA, FTC, and PEN A on human neuroblastoma SH-SY5Y cells were evaluated by the MTT assays over 24 h, 48 h, and 72 h. Fig. 2 shows the time- and concentration-dependent effect in cell viability after exposure to each mycotoxin individually, while IC_{50} values are shown in Table 2. No IC_{50} value was determined after 24h for any of the compounds assayed; however, after 48 h of exposure, IC_{50} values were determined at 2000 ± 3,1 µM and 18.5 ± 5.3 µM for AA and PEN A, respectively and after 72 h of exposure, the IC_{50} values were 1250 ± 4.5 µM and 14 ± 4.1 µM for AA and PEN A, respectively. According to the IC_{50} values obtained, PEN A showed the highest cytotoxic effect on SH-SY5Y cells (Table 17).

a) b) c)

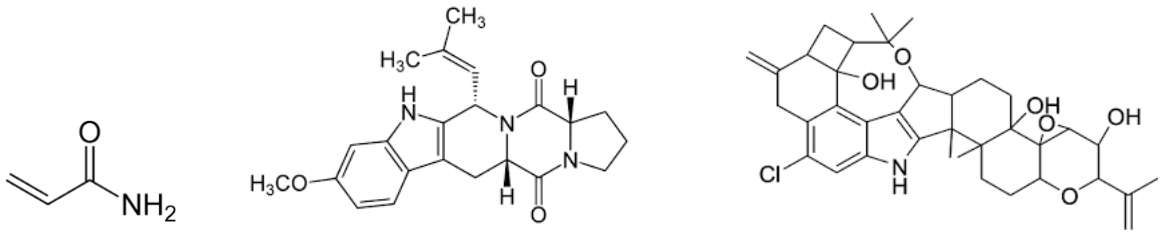


Figure 4. Chemical structures of the mycotoxins (a) AA, (b) FTC, and (c) PEN A.

Table 17. Medium inhibitory concentration (IC_{50} SD) of AA, FTC and PEN A in SH-SY5Y cells after three times of exposure by MTT assay. Three independent experiments were performed with eight replicates each.

Compounds tested	**IC_{50} ± SD (µM)**		
	24h	48h	72h
AA	NA	2000± 5.6	1250 ± 4.5
FTC	NA	NA	NA
PEN A	NA	18.5 ± 5.3	14 ± 4.1

NA: not achievable

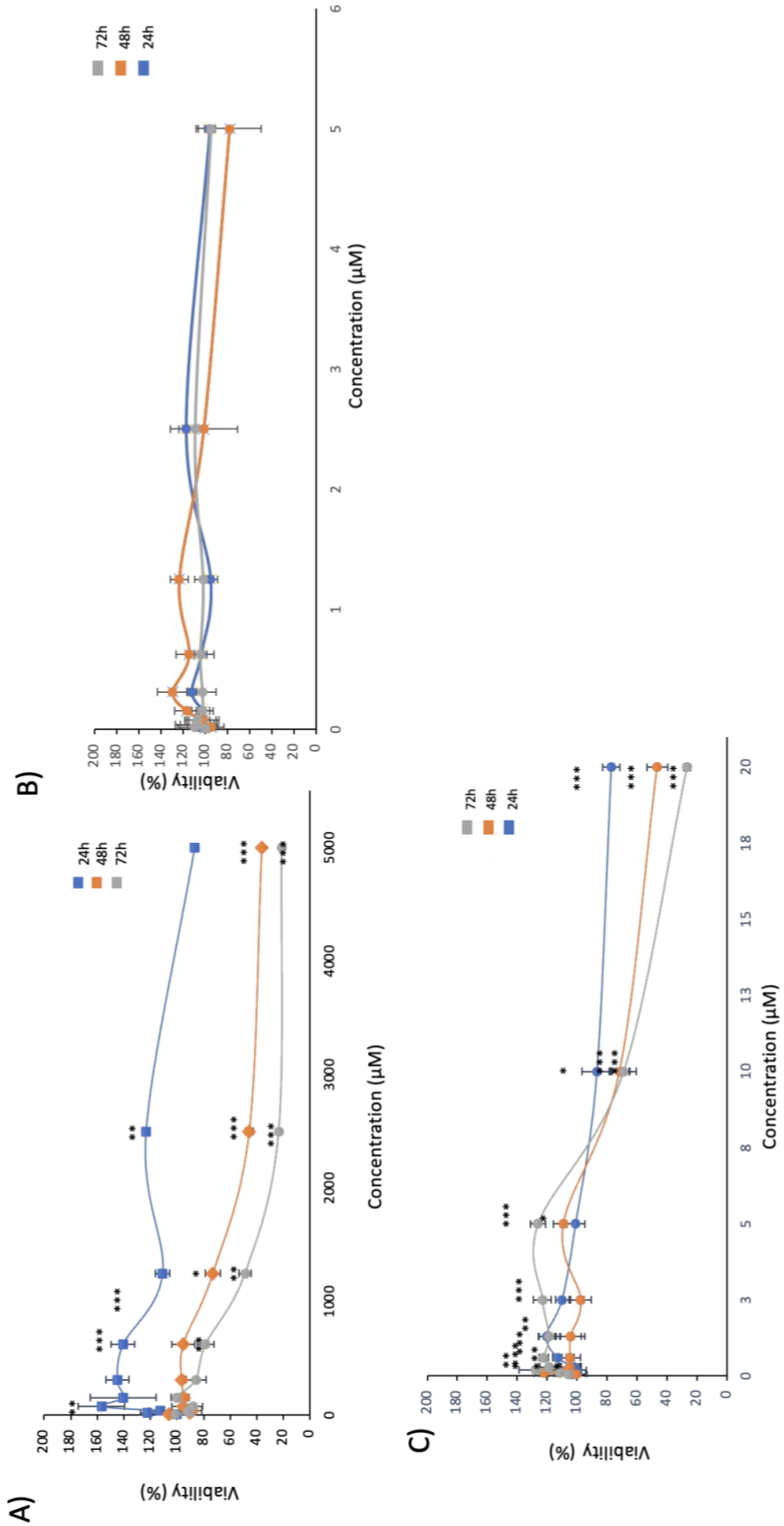


Figure 5. Concentration-effect curves obtained after (a) AA (b) FTC, and (c) PEN A, in SH-SY5Y cells after 24 h, 48 h and 72h of exposure by MTT assay. All values are results of two independent experiments with 8 replicates and expressed as mean ± SD. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

4.1.3.2. *Cytotoxicity effects of combined exposure to FTC, PEN A and AA*

The cytotoxic effect of binary combinations of AA, FTC, and PEN A on SH-SY5Y cells was evaluated by the MTT assays over 24 h, 48 h, and 72 h. The dose–response curves of the binary combinations are shown in Figure 6. It can be observed a concentration-dependent decrease in SH-SY5Y cell viability upon binary treatment for all the three times assayed. The [AA + FTC] combination decreased cell proliferation after 24 h of exposure by 1.7 % and 11 % at the highest concentration tested with respect to AA and FTC tested individually, respectively (Figure 6 a.1). After 48 h of exposure, the highest concentration of the combination augmented cell proliferation by 58.8 % and 43.1 % with respect to AA and FTC tested individually, respectively (Figure 6 a.2). For the 72 h exposure, cell viability augmented 56.3 % respect to AA, also, at 72 h of exposure, the combination decreased cell proliferation by approximately 18 % with respect to FTC individually tested (Figure 6 a.3). The [AA + PEN A] combination at the highest concentration induced a decrease in cell proliferation at 24 h of exposure of 16.2 % with respect to the effect of AA tested individually and 3.2 % with respect to the PEN A effect (Figure 6 b.1). After 48 h, the decrease in cell proliferation was 11.8 % with respect to that measured for AA and 3.9 % with respect to that measured for PEN A exposure (Figure 6 b.2). After 72 h of exposure, the viability augmented 35.2 % with respect to AA and 34.3 % with respect to PEN A (Figure 6 b.3). Similar effects were seen at 24h of exposure to [FTC + PEN A] (Figure 6 c.1), in which cell viability decreased 19 % respect FTC when tested individually and augmented 3.3 % respect PEN A. For 48h of exposure same tendency was observed in which cell viability decreased 18 % respect to FTC individual results and augmented 3.3 % respect PEN A (Figure 6 c.2). For the 72h exposure the mix of FTC and PEN A showed a decrease of 49 % in the viability respect FTC individually and an increase of 24.3 % if we compare the results to PEN A individual measures (Figure 6 c.3). Fig. 4 shows the dose–response curves for the tertiary combination of AA + FTC + PEN A at 24 h, 48 h, and 72 h of exposure in SH-SY5Y cells. At 24 h of exposure, cell proliferation decreased by 22 %, 31 % and 12.2 % compared to cells exposed to AA, FTC, and PEN A alone (Figure 7a). After 48 h of exposure, a significant reduction in cell proliferation, corresponding to 3.2 % and 13.2 %, was observed when compared

with AA, PEN A alone, and a reduction of 68 % was observed with respect to FTC alone (Figure 7b). At 72 h the combination of AA + FTC + PEN A showed a decrease of 68 % and 1 % compared to FTC and PEN A respectively at the highest concentrations and 52 % and 12 % at the second highest concentration (Figure 7c). Cytotoxicity after 24 and 48 h of incubation decreased in the following order: AA + PEN A + FTC > AA + PEN A > PEN A + FTC > AA + FTC. After 72 h of incubation, the ranking was AA + PEN A + FTC > PEN A + FTC > AA + PEN A > AA + FTC.

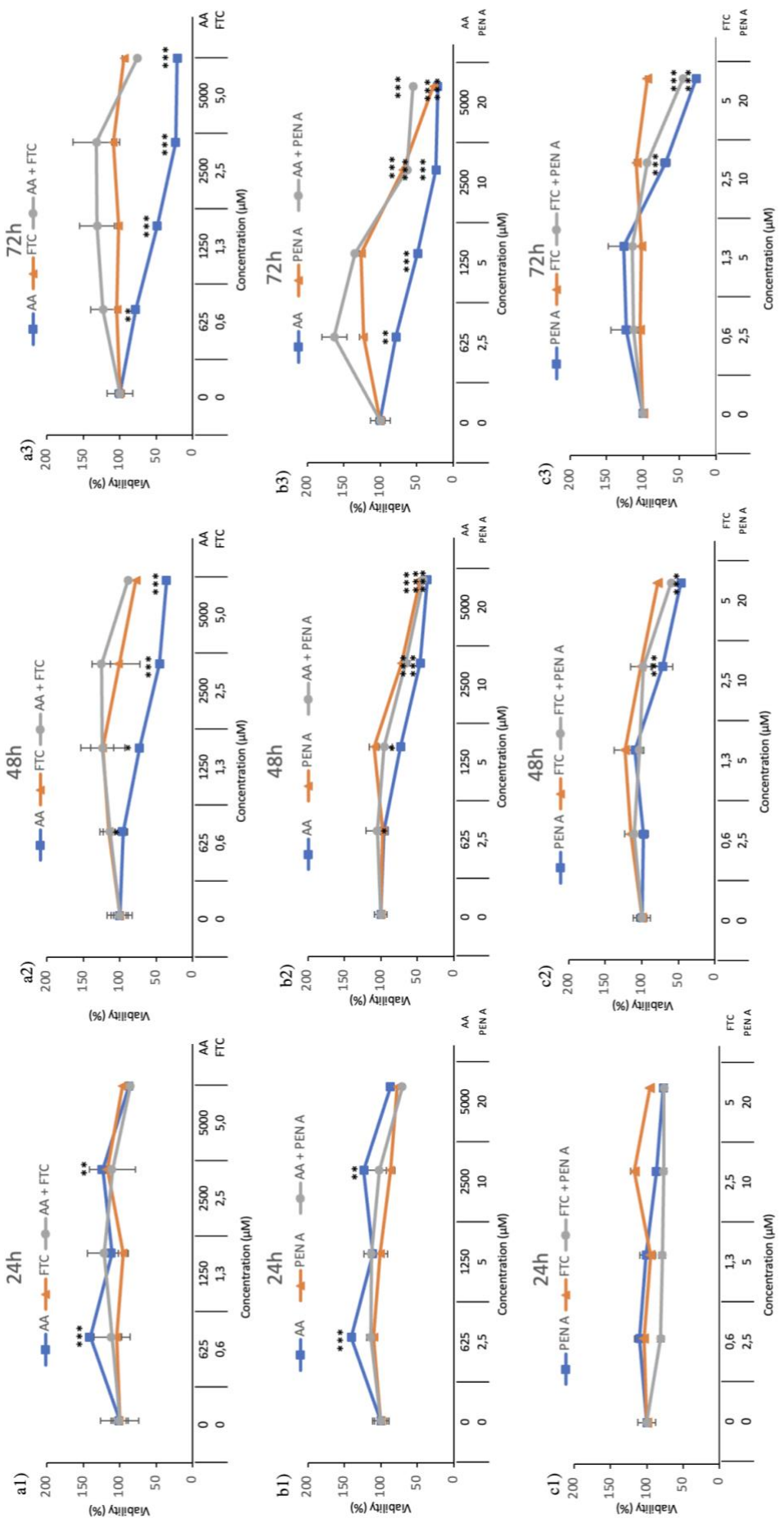


Figure 6. Cytotoxicity of the mycotoxin combinations of AA + FTC (a), AA + PEN A(b), and FTC + PEN A(c) at 24 h (a.1, b.1, and c.1), 48 h (a.2, b.2, and c.2) and 72 h (a.3, b.3, and c.3). All values are the results of three independent experiments with eight replicates and are expressed as mean ± SD; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

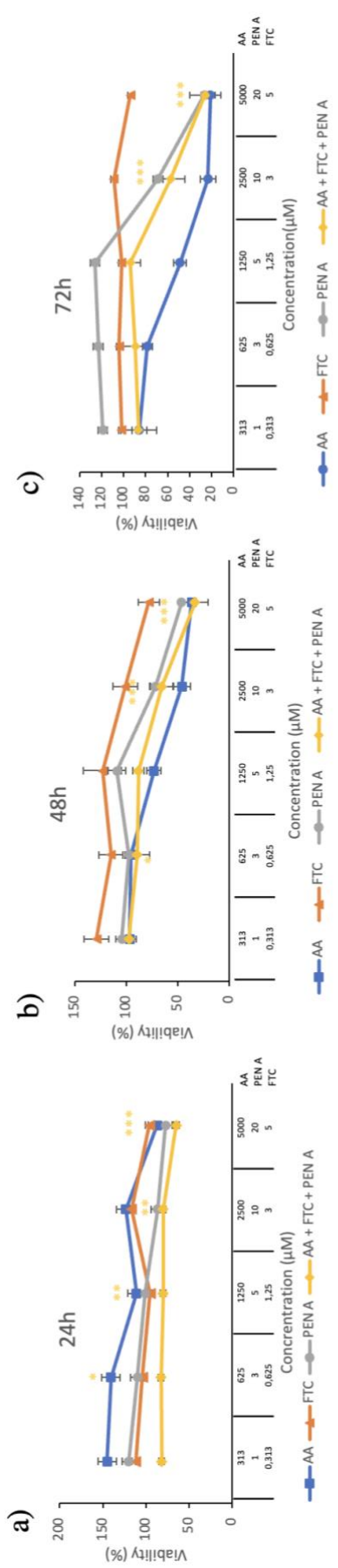


Figure 7. Cytotoxicity of the compound's combination AA + FTC + PEN A (1000:4:1) at 24h (a), 48h (b) and 72h (c). All values are results of three independent experiments with 8 replicates and expressed as mean ± SD. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

4.1.3.3. Isobologram analysis

The isobologram analysis was used to determine the type of interaction between AA, FTC, and PEN A. The values of the parameters Dm, m, and r of the double and triple combinations, as well as of the mean CI are shown in Table 18. The IC_{50}, IC_{75}, and IC_{90} are the doses required to inhibit proliferation at 50 %, 75 %, and 90 %, respectively. These CI values were calculated automatically by the computer software CalcuSyn. The CI fractional effect (fa) curves for AA, FTC, and PEN A combinations in SH-SY5Y cells are shown in Fig. 5. Additive for all concentrations of the AA + FTC (1000:1) mixture after 24 and 48 h of exposure was demonstrated (Fig. 8 a1, 8.a2); however, after 72 h of exposure, a synergic effect for the AA + FTC combination was observed (Fig. 8 a3, Table 18). The AA + PEN A (250:1) mixture showed additive at all concentrations after 24, 48 and 72 h of exposure (Fig. 8 b, Table 18). The mixture of FTC + PEN A showed additive effect at all concentrations assayed and at all times of exposure except 48 h at IC_{50} that showed a moderate synergism at low concentration (Fig. 8 c2, Table 18). The tertiary mixture, after 24 h of exposure, showed additive effect at all concentrations assayed, while after 48 h, it showed synergism and after 72 h, additive effect at all concentrations assayed (Fig. 8 d, Table 18)

Table 18. The parameters Dm. m. and r are the antilog of x-intercept, the slope, and the linear correlation of the median-effect plot, which means the shape of the dose–effect curve, the potency (IC_{50}), and the conformity of the data to the mass action law, respectively. Dm and m values are used for calculating the combination index (CI) value (CI < 1, =1, and >1 indicate synergism, additive, and antagonism effect, respectively. CI50, CI75, and CI90 are the doses required to inhibit proliferation at 50%, 75%, and 90%, respectively. CalcuSyn software automatically provided theses values.

Compound	Time (h)	*Dm* (μM)	*m*	*r*	CI Values					
					CI_{50}		CI_{75}		CI_{90}	
AA	24	2750.00	1.31	0.95185						
	48	2000	0.94135	0.98729						
	72	1250	0.71213	0.92624						
FTC	24	620272	2.02	0.94444						
	48	1436564	2.1	0.87849						
	72	2167388	2.11	0.94491						
PEN A	24	51	1.32	0.97312						
	48	14	1.6	0.97468						
	72	18.5	2.08	0.96178						
AA + FTC	24	21389	1.44	0.80329	0.76 ± 0.85	Add	0.9 ± 1	Add	1.1 ± 1.3	Add
	48	17310	1.26	0.94995	0.86 ± 0.33	Add	0.8 ± 0.35	Add	0.82 ± 0.39	Add
	72	664782648	1.79	0.9275	0.06 ± 0.33	Syn	0.03 ± 0.35	Syn	0.02 ± 0.39	Syn
AA + PEN A	24	11332	1.58	0.95185	1.28 ± 1.43	Add	1.12 ± 1.52	Add	0.98 ± 1.6	Add
	48	3750	1.92	0.96835	3.1 ± 1.38	Add	2.4 ± 1.29	Add	1.9 ± 1.27	Add
	72	2576	1.92	0.9572	0.55 ± 3.2	Add	0.29 ± 2.11	Add	0.2 ± 1.75	Add
FTC + PEN A	24	254	1.93	0.95	0.67 ± 1.16	Add	0.72 ± 1.54	Add	0.76 ± 2.03	Add
	48	257	1.93	0.969	0.33 ± 0.47	Syn	0.39 ± 0.69	Add	0.45 ± 0.99	Add
	72	257948	1.9	0.95635	0.15 ± 1.5	Syn	0.159 ± 1.9	Syn	0.17 ± 2.5	Add
AA + FTC + PEN A	24	37346	0.41835	0.84557	0.68 ± 1.16	Add	0.72 ± 1.54	Add	0.76 ± 2.03	Add
	48	351348534	0.59555	0.91335	0.34 ± 0.47	Syn	0.39 ± 0.69	Add	0.45 ± 0.98	Add
	72	222957687	0.67129	0.95967	0.25 ± 1.5	Add	0.41 ± 1.9	Add	0.85 ± 2.5	Add

Add: additive, **Syn**; synergism

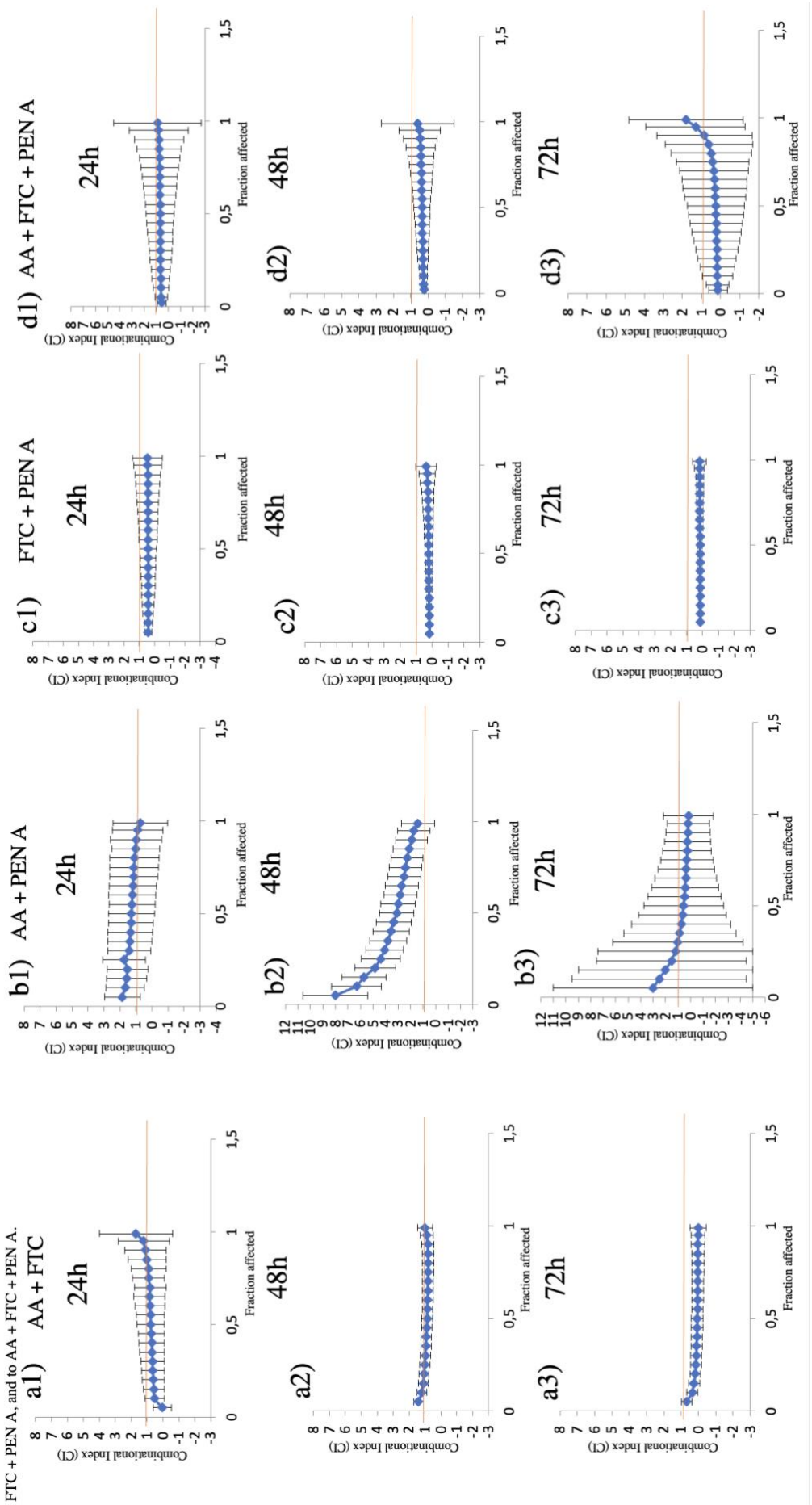


Figure 8. CI vs. fractional effect curve, as described by Chou and Talalay, for SH-SY5Y cells exposed to AA, FTC, and PEN A in binary and tertiary combinations. Each point represents the CI ± SD at a fractional effect as determined in our experiments. The line (CI = 1) indicates additivity, the area under this line indicates synergism, and the area above the line indicates antagonism. SH-SY5Y cells were exposed for 24, 48, and 72 h to AA + FTC, AA + PEN A and FTC + PEN A, and to AA + FTC + PEN A.

4.1.3.4. Cell cycle analysis in individual treatments

Results of cell cycle disturbances are represented in Figs. 9, 10 and 11 for individual and combined treatment, respectively. The results of 24 h showed that SH-SY5Y cells exposed to AA during 24 h at the highest and the lowest concentrations tested resulted in statistically significant differences compared to the control in phase G0/G1 ($p \leq 0.001$) (Figure 9 a.1). Effects observed correspond to a 23.4 % and 13.1 % decrease for 625 µM and 2500 µM of AA, respectively in G1/G0 phases, in the S phase a 21.5 % reduction was obtained at 625 µM and a 17.6 % increase for 2500 µM, regarding the M phase a 31.6 %, 21.5 % and 27.1 % decrease for 625 µM, 1250 µM and 2500 µM compared to control was observed, respectively. Similarly, this decrease was 14.6 % and 41.93 % at 625 µM and 2500 µM, respectively for G1/G0, 22.19 % and 70.93 % at 625 µM and 2500 µM, respectively for S phase, and 44.02 % and 49.17 % at 625 µM and 2500 µM, respectively for M phase was registered after 48 h of AA exposure, with a marked reduction at the higher concentrations in the S phase ($p \leq 0.01$) and in the M phase ($p \leq 0.001$) (Figure 9 a.2). At 72 h an increase of cell distribution in G0/G1 phase was observed (Figure 9 a.3). Regarding FTC, at 24 h a decrease in cell number was observed in all phases at the concentration assayed (Figure 9 b.1); while after 48 h a 15.93 % decrease was observed at 5 µM in the G1/G0 ($p \leq 0.01$) (Figure 9 b.2). At 72 h no significant changes were observed respect to the control (Figure 9 b.3). Regarding individual treatment of PEN A at 24 h of exposure, a 30.94 % and 67.79 % diminution was observed at 10 µM and 20 µM, respectively in the G0/G1 phase, 43.51 %, 44.84 % and 53.17 % at the S phase at 5 µM, 10 µM, and 20 µM, respectively (Fig. 7 c.1) and a 38.16 % reduction at the M phase at 20 µM. Furthermore, after 48 h of exposure a 90.16 % decrease was observed at 20 µM, respectively in the G1/G0, 82.14 % at 20 µM in the S phase and 66.62 % at 20 µM at the M phase. Also, at 10 µM there was a 63.52 % decrease in the G1/G0 phase (Figure 9 c.2). At 72 h of exposure, a 72.45 % and 95.78 % diminution at G1/G0 phase, 61.64 % and 97.04 % diminution at S phase, 35.86 % and 95.82 % diminution were observed at M phase studied at 10 µM and 20 µM, respectively (Figure 9 c.3).

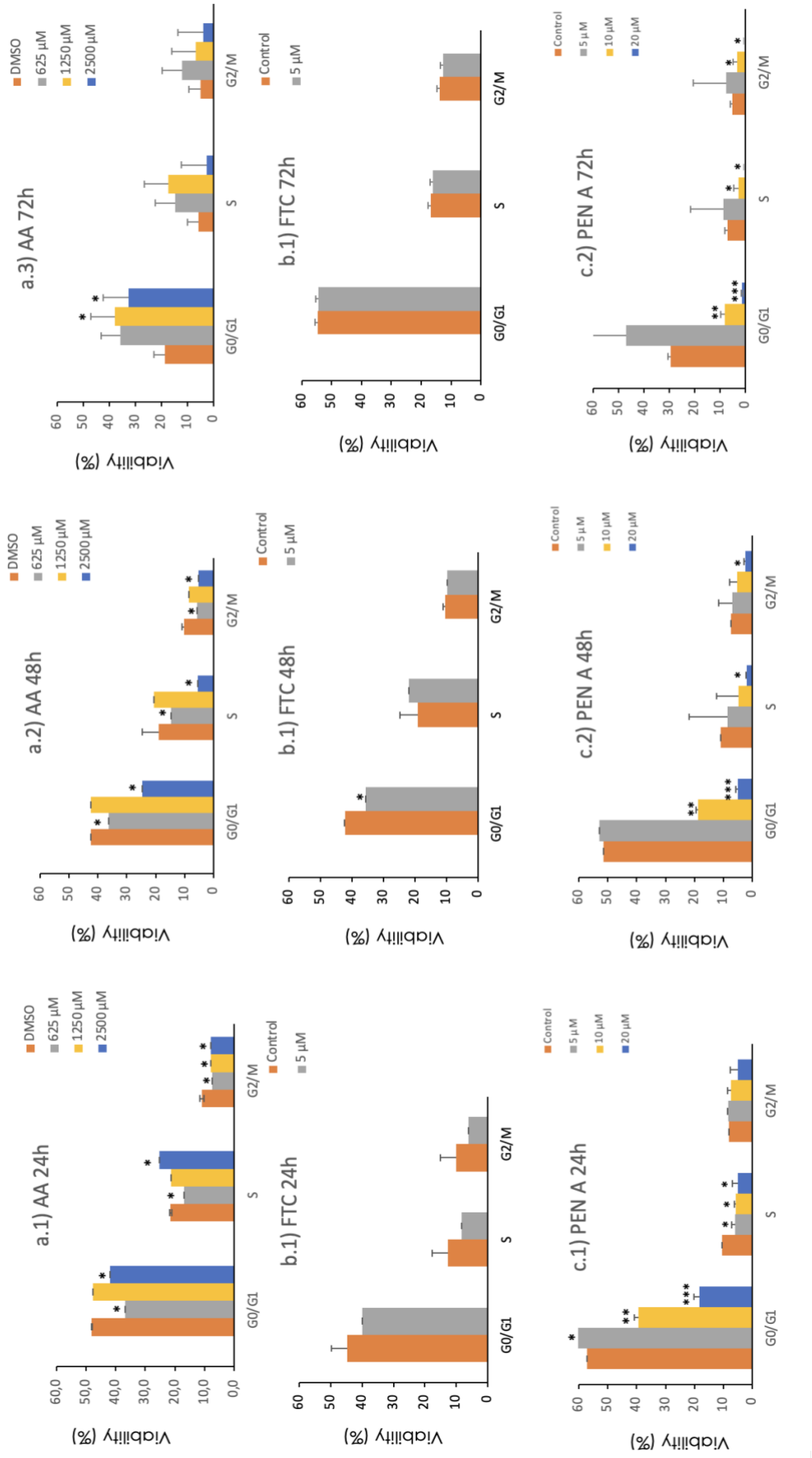


Figure 9. Cell cycle phase distribution obtained after the exposure in SH-SY5Y cells of AA, after 24h (a.1) 48h (a.2) and 72h (a.3), FTC after 24h (b.1) 48h (b.2) and 72h (b.3) and Pen A after 24h (c.1) 48h (c.2) and 72h (c.3) of exposure. All values are results of two independent experiments with 8 replicates and expressed as mean ± SD. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

4.1.3.5. Cell cycle analysis in combination treatments

Regarding combined treatment [AA + FTC] a statistically significant decrease of 67.78 % ($p \leq 0.01$) and 30.94 % ($p \leq 0.05$) was observed in the G0/G1 phase at [2500 + 5] µM and [1250 + 2,5] µM concentrations respectively and an increase of 22.16 % ($p \leq 0.05$) at the lowest concentration assayed [625 + 1,25] µM. Likewise, a decrease of 53.17 %, 44.84 % and 43.52 %, at [2500 + 5] µM, [1250 + 2,5] µM and [625 + 1,25] µM in the S phase was perceived after 24 h treatment (Figure 10 a.1). After 48 h, a decrease of 90.16 %, 82.14 % and 66.62 % in the G0/G1, S and in the G2/M phase respectively was observed at [2500 + 5] µM ($p \leq 0.01$, $p \leq 0.05$ and $p \leq 0.05$ respectively) (Figure 10 a.2). At [1250 + 2,5] µM a decrease of 63.51 % was observed at the G0/G1 phase ($p \leq 0.05$). At 72 h of exposure, a significantly diminution of 95.79 %, 97.04 % and 95.82 % ($p \leq 0.01$, $p \leq 0.05$ and $p \leq 0.05$ respectively) was observed at G0/G1, S and G2/M phases, studied at the highest concentrations [2500 + 5] µM, while at [1250 + 2,5] µM a decrease of 72.45 % was observed in the G0/G1 phase ($p \leq 0.05$) (Figure 10 a.3). Concerning the results obtained after 24 h of exposure the combined treatment of AA and PEN A, showed a statistically significant decrease of 21.12 %, 11.45 % and 9.89 % at [2500 + 20] µM, [1250 + 10] µM and [625 + 5] µM concentrations tested ($p \leq 0.05$ for all cases) (Figure 10 b.1). Similarly, after 48 h of exposure to the combined treatment a diminution of 58.01 %, 48.47 % and 15.25 % ($p \leq 0.01$, $p \leq 0.01$ and $p \leq 0.05$, respectively) in the G0/G1 phase was observed at [2500 + 20] µM, [1250 + 10] µM and [625 + 5] µM, respectively. Moreover, at 48 h of exposure, there was a 55.13 % decrease in G2/M phase ($p \leq 0.05$) (Figure 10 b.2). With respect to the combined treatment [FTC + PEN A] after 24 h of exposure, a diminution of 5.08 % at [20 + 5] µM ($p \leq \_0.05$) and an augmentation of 7.57 % at [10 + 5] µM ($p \leq 0.05$) in the G1/G0 phase was observed (Figure 10 c.1). At 48 h of exposure a marked diminution in G0/G1 phase of 91 % at [20 + 5] µM was observed, nevertheless, at [10 + 5] µM and [5 + 5] µM an increase of 12 % ($p \leq 0.05$) and 114.5 % ($p \leq 0.01$) was observed, respectively; respecting the S phase there was an decrease of 88.25 %, 556.14 % and 52.87 % at [20 + 5] µM, [10 + 5] µM and [5 + 5] µM, respectively ($p \leq 0.05$ at all cases). Regarding the G2/M phase a decrease of 93.56 %, 44.13 % and 29.92 % at [20 + 5] µM, [10 + 5] µM and [5 + 5] µM, respectively ($p \leq 0.01$, $p \leq 0.01$ and $p \leq 0.05$,

respectively). So, the G0/G1 phase increased at the lowest concentration and decreased at the higher concentration, followed by a decrease at all concentrations in the S phase, equally in the G2/M phase statistically significance decrease at all concentration was obtained (Figure 10 c.2). For 72h of exposure cell distribution in the G0/G1 phase diminished 93.18 % at [20 + 5] μM ($p \leq 0.01$) and 54.98 % at [10 + 5] μM ($p \leq 0.05$) and increased 18.91 % ($p \leq 0.01$) for the lowest concentration [5 + 5] μM, this was accompanied by a reduction of 91.51 % and 76.02 % at [20 + 5] μM and [10 + 5] μM, respectively in cells population in S and a decrease of 90.83 % and 60.55 % in G2/M phases at the highest concentrations assayed ($p \leq 0.05$ for all cases) (Figure 10 c.3). Ultimately, tertiary combination of AA + PEN A + FTC is shown in Figure 11. After 24 h of exposure a considerable decline in G0/G1, S and G2/ M phase cells population by 42.70 %, 49.01 % and 64.6 %, respectively ($p \leq 0.05$ for all cases) at [5000 + 20 + 5] μM, (Figure 11 a.1). After 48 h of exposure, the population of cells in G0/G1 and S phases increased significantly at the highest concentration assayed ([5000 + 20 + 5] μM) by 64.34 % and 61.03 %, respectively, while a significant increase in G0/G1 phase was observed at [2500 + 10 + 5] μM by 37.93 % (Figure 11 b.1). At 72 h an increase of 204.23 %, 315.42 % and 327 % at [5000 + 20 + 5] μM, [2500 + 10 + 2.5] μM and [1250 + 5 + 1.25] μM, respectively ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively) in the G0/ G1 phase (Figure 11 c.1).

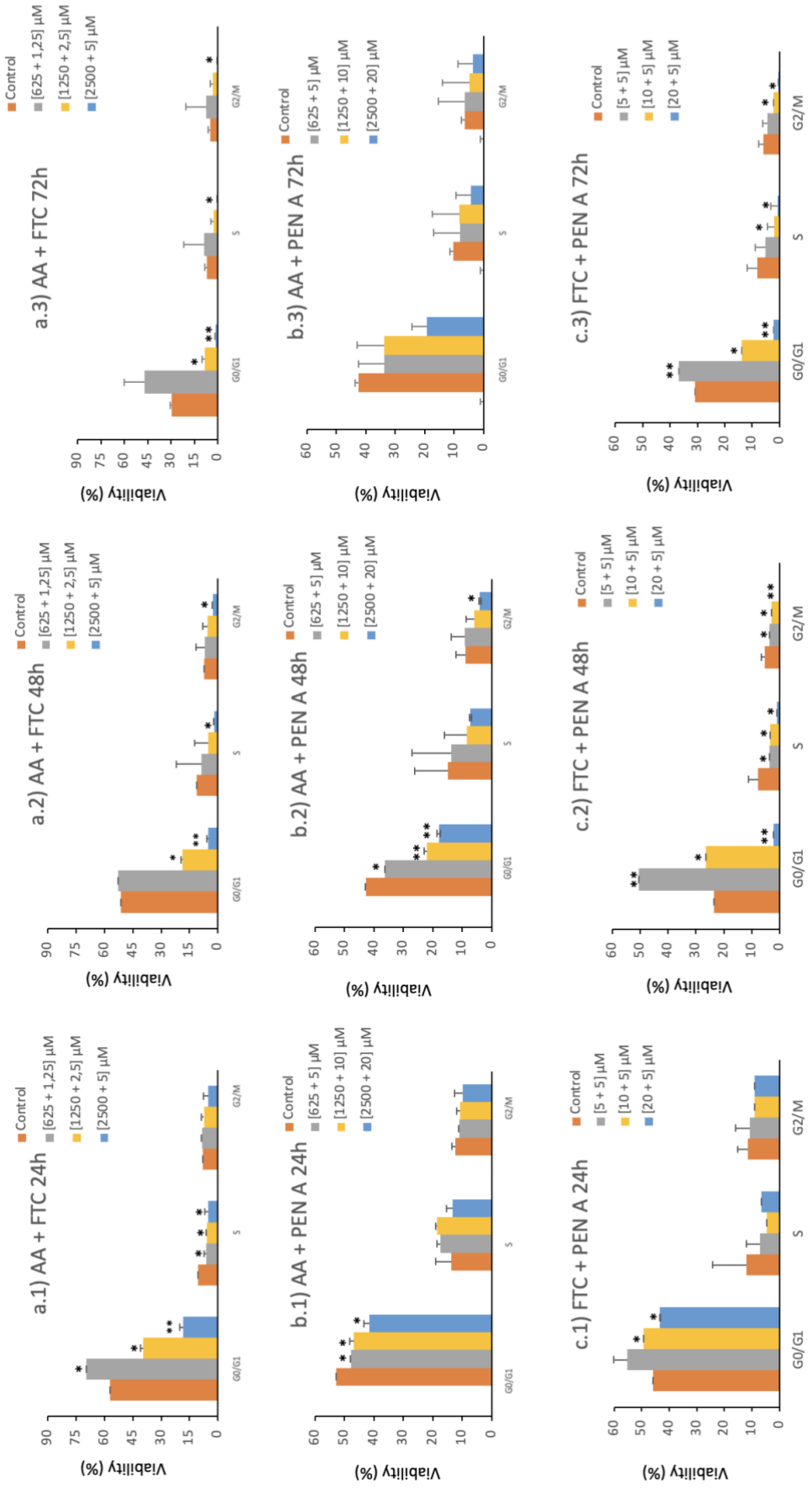


Figure 10. Cell cycle phase distribution in SH-SY5Y cells after exposure to AA + FTC, AA + Pen A, and FTC + Pen A for 24 h (a.1, b.1, c.1), 48 h (a.2, b.2, c.2), and 72 h (a.3, b.3, c.3), respectively. Data represent the mean ± SD of two independent experiments with eight replicates each. Statistical significance:$p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

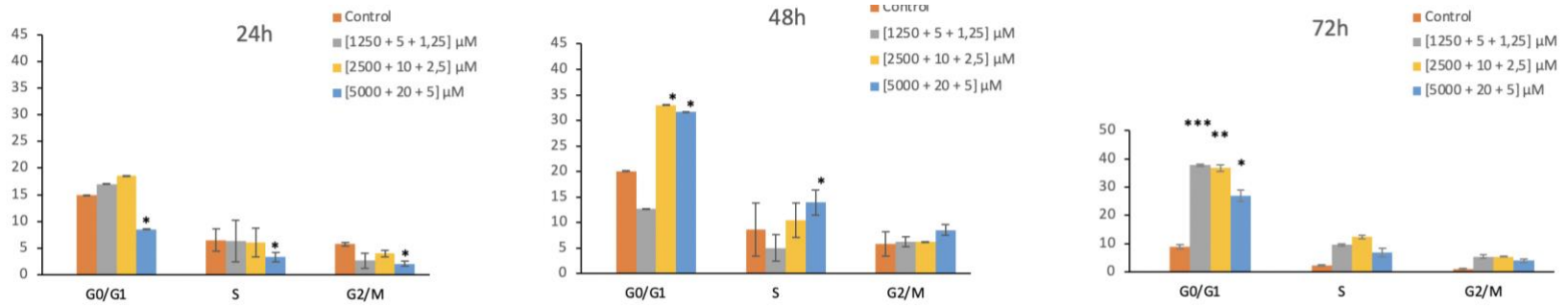


Figure 11. Cell cycle phase distribution obtained after the exposure in SH-SY5Y cells of AA, FTC and PEN A, after 24h (a.1) 48h (a.2) and 72h (a.3) of exposure. All values are results of two independent experiments with 8 replicates and expressed as mean ± SD. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

4.1.4. Discussion

Among the three compounds, the effect of AA in SH-SY5Y has been widely studied (Forsby, 2011; Forsby et al., 1995; Hartley et al., 1997; Okuno et al., 2006; Sumizawa and Igisu, 2007, 2008, 2009), while no reports have been found on the study of FTC and PEN A in an in vitro culture of neuronal cells. In the present work, for the first time it has been reported the individually study of FTC and PEN A in SH-SY5Y cells as well as the AA, FTC, and PEN A combined effect in human neuroblastoma SH-SY5Y cells in relation to time mixture and compound exposure. AA has been classified as group 2A by the IARC. AA and its metabolite glycidamide form covalent adducts with DNA in mice and rats. In addition, AA causes genetic mutations and chromosomal aberrations in rodent somatic cells in vivo, cultured cells in vitro, and mouse germ cells.

Regarding cytotoxicity, several authors studied AA, in SH-SY5Y cells reporting effects for doses (0–10 mM) and exposure time (from 4 to 72h) similar to ours (Song et al., 2017; Yan et al., 2019, 2022). FTC showed no cytotoxic effect as shown in other studies in which they assayed mitoxantrone-selected human colon carcinoma cell line (S1-M1-3.2), for 72 h, individually and in combination to other compounds, it was shown that it could increase the drug accumulation in resistant cells, likely blocking the action of a putative drug transporter protein and allowed the cytotoxic agent to reach lethal levels (Sridhar K. Rabindran et al., 1998) According to the IC_{50} values of single compounds, PEN A was the most cytotoxic mycotoxin compared to the other compounds assayed individually, similarly to the values obtained by (Bünger et al., 2004) who studied the cytotoxicity of PEN A individually, among other mycotoxins, in 4 different types of cells L-929 A-549 Neuro-2a and Hep-G2, with IC_{50} values of 34.2, 20.5, 10.7 and 21.1 µM, respectively. Compared to our results it is lower IC_{50} values, due to the type of cells used and lower concentrations range were assayed. According to the double combination, it was revealed that the presence of two compounds attenuated the cytotoxic potential in SH-SY5Y cells, as shown by the higher IC_{50} values. In SH-SY5Y cells, almost all the combinations tasted increased cell viability more than individual compounds, except for AA and PEN A double, and the triple combination. No studies about the interaction of AA, FTC and PEN A combination have been found,

nevertheless several studies have stated the importance of studying the interactions between AA combined to other mycotoxin, Ochratoxin A (OTA) in human kidney cells (HK-2), liver cells (HepG2) and Caco2 cells, (Pyo et al., 2020; Su et al., 2023).

Therefore, the potential mechanisms of AA-, FTC, and PEN A-induced neurological dysfunction must yet be explored. Cytotoxicity of AA and PEN A in SH-SY5Y cells either in single or combined treatment can interfere in cell proliferation; subsequently, cell cycle alterations either individually or combined were assayed. The cell cycle is the series of events in which cellular components are doubled, and then accurately segregated into daughter cells. In eukaryotes, DNA replication is confined to a discrete synthesis or S-phase, and chromosome segregation occurs at Mitosis or M-phase. In general terms, for all cases a decrease in G0/G1 was detected, revealing induction of cell death at the concentrations assayed. In accordance with the results of cytotoxicity obtained. For AA, a marked decrease in de G0/G1 phase as showed in other studies could be due to the fact that (Sumizawa and Igisu, 2007) flow cytometric analysis showed increase of population in sub-G1 phase in SH-SY5Y cells exposed to AA.

Furthermore, in the same study they analyzed the caspase-3 activation and how its suppression by Z-VAD-fmk diminished the cytotoxicity. These results obtained by Sumizawa and Igisu. (2007) indicate that apoptosis may be involved in the genesis of toxicity of AA in SH-SY5Y cells. Thus, to our knowledge, our results in the cell cycle disruption might be to the fact that AA induces apoptosis as Sumizawa and Igisu. (2007) stated that AA can activate caspase-3 and cause apoptosis in neuronal cells. In accordance with the results from cell cycle analysis presented for SH-SY5Y cells, after 24 h of exposure to FTC, cell proliferation was arrested remarkably in G0/G1 phase by the mycotoxin in comparison with non-treated cells. More studies are needed in other to fully understand the mechanisms of action of FTC. Regarding the cell cycle alteration induced by PEN A, we can also observe a diminution in the cell distribution in G0/G1, this can also be caused by the capacity of PEN A to induce apoptosis. Some studies, have state that PEN A can upregulate the TNF-α _and given that TNF-α _is a major cytokine known to induce cancer cell death through sustained JNK-activation, it can be

concluded that the antiproliferative activity of PEN A is associated, in part, with the upregulation of TNF-α and subsequent activation of apoptotic cell death, as stated in other studies (Balkwill, 2009; Goda et al., 2018; Lin and Wang, 2008). This study provides critical insights into the cytotoxic effects of AA, FTC, and PEN A on SH-SY5Y cells. The findings emphasize the importance of studying mycotoxins individually and in combination, as well as understanding their impact on cell cycle progression due to the fact that humans are exposed to a large number of mycotoxins simultaneously. These insights have implications for food safety standards and highlight the potential risks associated with exposure to these food contaminants in a more realistic scenario of mycotoxins with other food contaminants. However, more research in this area is essential for a comprehensive understanding of their effects on human health and further studies must be conducted to determine the cytotoxic mechanism of action of PEN A and FTC, as well as the interaction between AA, FTC and PEN A.

4.1.5. Conclusions

In conclusion, AA and PEN A were cytotoxic, especially, the treatment with PEN A alone presented the highest cytotoxic potency compared to treatments with the other compounds assayed (AA and FTC). The main type of interaction detected between compounds for all combinations assayed was additive. Moreover, AA and PEN A disrupted the cell cycle of SH-SY5Y cells, among all compounds assayed, our results demonstrate that PEN A possessed the highest potential in disturbing cell cycle progression by activating and/or arresting cells in G0/ G1 phase, suggesting the capacity to induce apoptosis in SH-SY5Y cells.

CHAPTER II: 4.2. Study of oxidative stress induced by individual and combined exposure to acrylamide, penitrem A, and 3-acetyldeoxynivalenol in SH-SY5Y human neuroblastoma cells

4.2.1. Introduction

Oxidative stress is a crucial factor in the pathophysiology of various diseases (García-Sánchez et al., 2020). It occurs when the production of reactive oxygen species (ROS) surpasses the cells antioxidant defences, causing lipid peroxidation (LPO), protein oxidation, and DNA fragmentation, leading to cellular damage and mutations that can contribute to several pathologies (Burton and Jauniaux, 2011). This imbalance can result in significant cellular dysfunction and is implicated in various chronic diseases and health conditions, such as, Alzheimer's disease, respiratory distress syndrome, toxicity, and aging(García-Sánchez et al., 2020; Jomova et al., 2023). Therefore, the importance of oxidative stress lies in its widespread impact on cellular health and its role in the development of numerous chronic diseases. To have a comprehensive view of cellular responses to oxidative stress and the overall redox status within the cells the study of molecular markers indicative of oxidative stress, such as ROS, LPO, and the glutathione (GSH/GSSG ratio) are a good target.

Reactive oxygen species (ROS) serve dual roles, acting as essential signaling molecules but causing harm when excessive (Milkovic et al., 2019). Literature reveals that studying ROS interactions with cellular defences is crucial, especially regarding food contaminants (e.g., mycotoxins, pesticides) from harvest or processing (Jomova et al., 2023). Lipid peroxidation (LPO) in brain models is key due to the brain’s vulnerability to oxidation, leading to neurotoxic aldehydes linked to several diseases (Reed, 2011). One example is the case of oxidized phospholipids which indicate inflammation and are tied to neurological conditions (Deamer, 2017). Lastly, glutathione’s reduced-to-oxidized ratio (GSH/GSSG) reflects redox status, decreasing under oxidative stress, signaling potential cellular damage (Vašková et al., 2023). Overall, oxidative stress is a common mechanism through which contaminants induce cell damage, impacting processes like DNA repair and cell survival (Lagunas-Rangel et al., 2022).

When pointing the contaminants where these markers have been used the number of publications is very high (Mézes et al., 2021). On one hand, for food processing contaminates like acrylamide (AA), a by-product of high-temperature cooking (Sebastià

et al., 2023), it has been stablished the ability to form adduct with reduced glutathione and increases in the production of hydrogen peroxide, leading to increased levels of LPO products and carbonyl content in the cerebral cortex and the cerebellum in rat (Allam et al., 2011; Lakshmi et al., 2012). On the other hand, natural contaminants such PEN A mycotoxin several metabolites have been found in liver extracts of mice (*in vivo*) after oral exposure to PEN A, similarly as *in vitro* exposure with primary rat hepatocytes and rat liver microsomes (Eriksen et al., 2010; Moldes-Anaya et al., 2009). PEN A is probably the main tremorgenic compound in *Penicillium*-infected food and feed commodities (González et al., 2003). PEN A affects the central as well as the peripheral nervous system. It blocks the high-conductance Ca^{2+}-activated potassium channels (BK) and impairs the GABAergic neurotransmission in the cerebellum (Eriksen et al., 2013a). The interest of mycotoxin 3-acetyldeoxynivalenol (3-ADON) strives in the fact that it is the acetylated analogue of the most commonly occurring trichothecene, deoxynivalenol (DON), derived from mold-contaminated food, it has been identified as inducers of oxidative stress in different cell lines (Juan-García et al., 2019).

Understanding the impact of natural and food processed contaminants on SH-SY5Y cells, a widely used human neuroblastoma cell line, provides crucial insights into the cellular responses to the potential neurotoxic substances. The importance relays not only in the individual effect of the food contaminants but in studying the interactions between them, understanding the potential health risks associated with consuming contaminated food with more than one substance. When multiple contaminants are present, they may interact in ways that could enhance (synergistic), diminish (antagonistic), or have an additive effect on their toxicity (Lagunas-Rangel et al., 2022). This knowledge is essential for developing effective strategies to mitigate these risks and protect human health.

SH-SY5Y cells serve as an invaluable model for neuronal cells, offering a model to explore the effects of toxic compounds on neural function. It has been studied that exposure to AA, and PEN A, triggers cytotoxicity in SH-SY5Y cells, leading to cellular

damage and dysfunction (Bridgeman et al., 2024); however, 3-ADON has not been studied yet in SH-SY5Y cells. The challenge lies in understanding the intricate mechanisms underlying the oxidative stress response to these compounds individually and when combined as happening in a real scenario in consumer's food intake. Therefore, investigating the impact of AA, PEN A, and 3-ADON on oxidative stress in SH-SY5Y cells, combined with the assessment of ROS, LPO, and GSH/GSSH, provides a comprehensive understanding of the implicated molecular mechanisms.

4.2.2. Material and methods

- Reagents (described in section 3.1.)

- Cell culture (described in section 3.2.)

- Cell treatment (described in section 3.3.)

- MTT assay (described in section 3.4.)

- *Intracellular ROS generation* (described in section 3.9.)

- *Lipid peroxidation assay* (described in section 3.10.)

- *GSH and GSSG determination* (described in section 3.11.)

- *Statistical analysis* (described in section 3.14.)

4.2.3. Results

4.2.3.1. Cytotoxicity effects of combined exposure to AA, PEN A, and 3-ADON

Figure 12 shows the time- and concentration-dependent effect of individual, and combinations tested. The cytotoxicity effects of AA, and PEN A individually and combined on human neuroblastoma SH-SY5Y cells is reported in Bridgeman et al., (2023); while the individual and combined effect of those with 3-ADON for the first time carried out is reported in Figure 12. In individual treatment IC_{50} values were obtained after 48 h and 72 h for 3-ADON (Figure 1a); while in combination treatment all exposure times reached IC_{50} values, except for [AA + 3-ADON] (Table 14). Regarding the decrease in cell viability, at 24h for the combinations [AA + 3-ADON] and [PEN A + 3-ADON] at the highest concentration tested the viability decreased 41 % *($p \leq 0.01$)* and 62 % *($p \leq 0.01$)* respectively, compared to the control (Figure 10b and 10c). Regarding the tertiary combination of AA + PEN A + 3-ADON, there was an 80 % *($p \leq 0.001$)* reduction of the viability at the highest concentration ([5000 + 20 + 4] µM) also at 24 h compared to the control (Figure 12d).

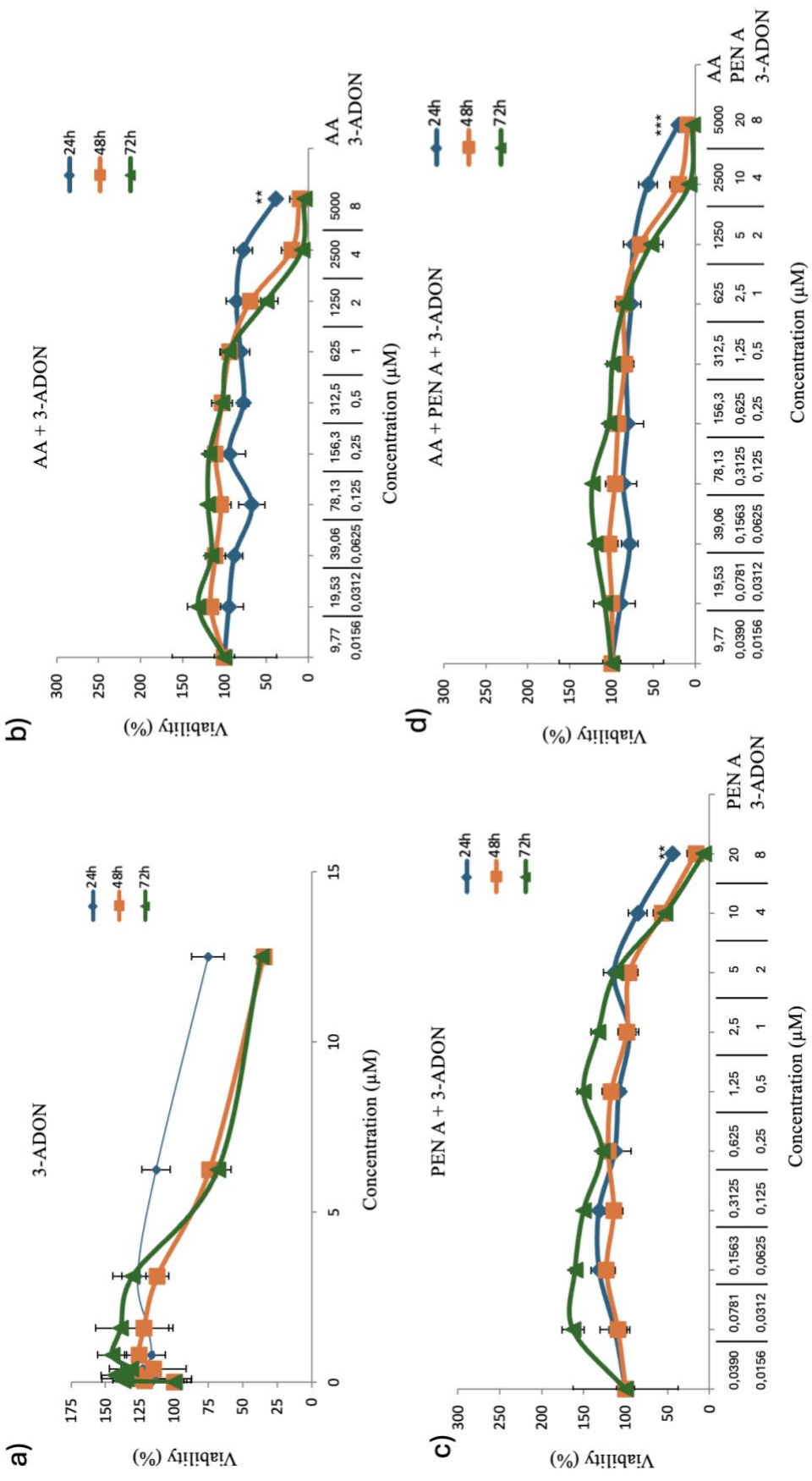


Figure 12. Concentration effect curves obtained after (a) 3-ADON (b) PEN A + 3-ADON, and (c) AA + 3-ADON (d) AA + PEN A + 3-ADON, in SH-SY5Y cells after 24 h, 48 h and 72h of exposure by MTT assay. All values are results of three independent experiments (n = 3) with 8 replicates and expressed as mean ± SD. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

4.2.3.2. ROS effects of individual exposure to AA, PEN A, and 3-ADON

The intracellular ROS generation in SH-SY5Y cells treated with AA, PEN A, and 3-ADON, individually or combined, at various concentrations over a 0–120 minute period is shown in Figure 13. The individual exposure to AA shows the same pattern at all times tested (Figure 13a): at higher AA concentrations higher ROS production is observed; concerning the lowest concentration, there is a significant difference to the control ranging from 6.85 to 14.47 % *($p \leq 0.001$)*. The exposure to 2.5 mM of AA reaches up an increment of 18.41 % *($p \leq 0.001$)* and at the highest concentration of AA (5 mM) increases the ROS production a 20.22 % *($p \leq 0.001$)* compared to the control (Figure 13a). However, differences over time were not detected.

Regarding the individual exposure to PEN A, at 10 µM concentration, there was significant changes compared to the control, at 30 and 90 minutes of exposure in which there was an increment of 5.15 and 3.56 % *($p \leq 0.05$),* respectively, compared to the control (Figure 11b). For the highest concentration tested (20 µM) a significant increase was observed at all times *($p \leq 0.001$)*, obtaining the most elevated level at 15 min with a rise of 24.63 % (Figure 13b).

Considering the individual treatment with 3-ADON a dose dependent manner increase was observed at all times of exposure (Figure 13c). At 3-ADON 2 µM concentration the highest result was observed at 45 min with an increase of 20.64 % *($p \leq 0.01$).* While at 4 µM the highest increase was obtained after 5 min with an increase in the ROS production of 26.90 % *($p \leq 0.001$)* (Figure 13c).

To sum up, the most significant increase in ROS production occurred with individual exposure to 3-ADON at 4 µM, which resulted in a rise after just 5 minutes, followed closely by the increase observed at 15 minutes with 20 µM of PEN A. Meanwhile, AA at 5 mM led to a maximum ROS increase, highlighting a concentration-dependent effect across all compounds.

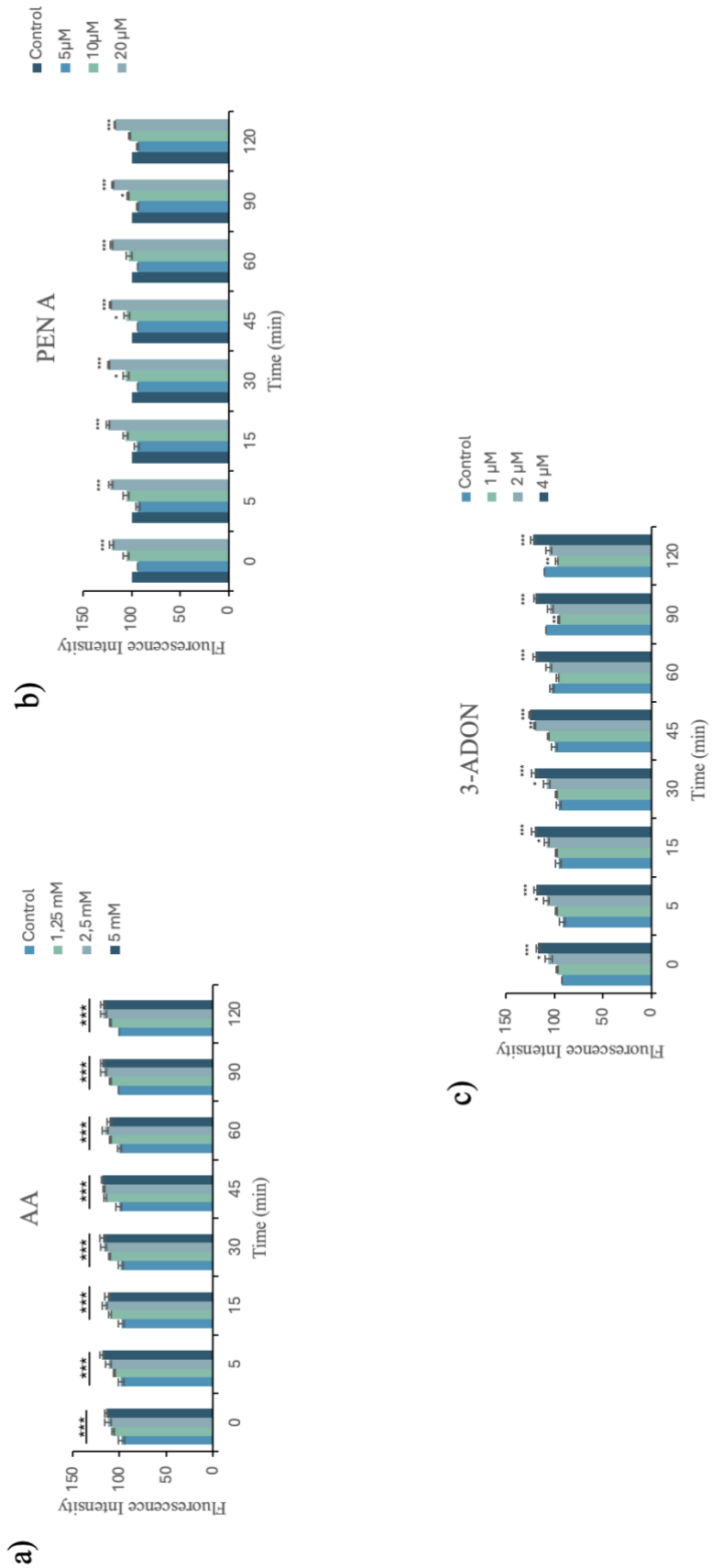


Figure 13. Time-dependent ROS-induced fluorescence in SH-SY5Y cells exposed to: a) AA at 1.25, 2.5, and 5 mM; b) Pen A at 5, 10, and 20 µM; and c) 3-ADON at 1, 2, and 4 µM. H_2DCFDA was added to SH-SY5Y cells and incubated for 20 minutes prior to compound exposure, which lasted from 0 to 120 minutes. Results are expressed as mean ± SEM (n = 2)

4.2.3.3. ROS effects of combined exposure to AA, PEN A and 3-ADON

The ROS effects of binary combinations of AA, PEN A, and 3-ADON on SH-SY5Y cells are shown in Figure 14. For [AA + PEN A] combination an increased in the ROS production was obtained after at 5 min by 10.99 % *($p \leq 0.01$)* at the highest concentration of [5000 + 20] µM (Figure 12a). Considering the combined treatment of [PEN A + 3-ADON] at time 0 of study there was a significant increase at all concentrations tested [5 + 1] µM, [10 + 2] µM and [20 + 4] µM of 22.79 % *($p \leq 0.001$),* 19.08 % (*$p \leq 0.001$),* and 15.87 % *($p \leq 0.001$),* respectively and at 5 min for the same concentrations a significant increase of 15.75 % *($p \leq 0.001$),* 13.08 % (*$p \leq 0.001$*), and 9.35 % (*$p \leq 0.001$*), respectively compared to the control was obtained (Figure 14c). For the tertiary combination [AA + PEN A + 3-ADON], at 60 min an increase of 8.24 % (*$p \leq 0.001$*) in the ROS production at the highest concentration of [5000 + 20 + 4] µM tested was observed, and a similar tendency was observed at 90 min and 120 min with an increment of 10.38 % (*$p \leq 0.001$*) and 12.92 % (*$p \leq 0.001$*), respectively (Figure 14d). In general terms the combinations tested reduced the ROS production compared to individual treatment in SH-SY5Y cells. The ROS production after 24 h of exposure increased in the following order: [AA + PEN A] > [AA + 3-ADON] > [PEN A + 3-ADON] > [AA + PEN A+ 3-ADON].

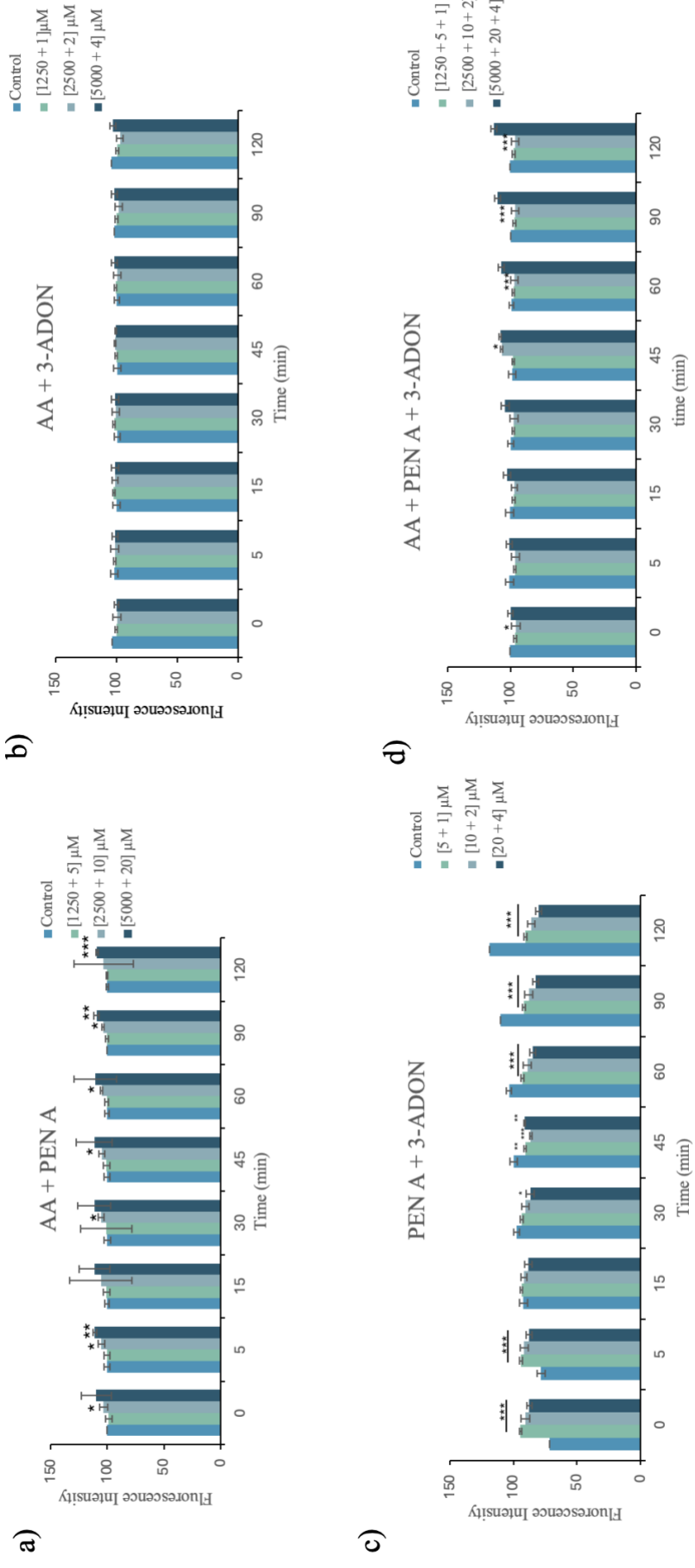


Figure 14. Time dependence of ROS-induced fluorescence in SH-SY5Y cells exposed to a) AA + PEN A at (1250 µM , 2500 µM, 5000µM) + (5 µM , 10 µM, 20 µM) , b) AA + 3-ADON (1250 µM , 2500 µM, 5000µM) + (1 µM, 2 µM, 4 µM), c) PEN A + 3-ADON (5 µM , 10 µM, 20 µM) + (1 µM, 2 µM, 4 µM) and d) AA + PEN A + 3-ADON at (1250 µM , 2500 µM, 5000µM) + (5 µM , 10 µM, 20 µM) + (1 µM, 2 µM, 4 µM). . The H2-DCFDA was added to SH-SY5Y cells and left for 20 min previously to the compounds addition from 0 to 120 min. Results are expresses as mean ± SEM (n = 2).

4.2.3.4. Lipid peroxidation assay of individual exposure to AA, PEN A, and 3-ADON

Results of LPO obtained after 24 h of exposure to AA, PEN A and 3-ADON are reported in Figure 15. For PEN A revealed significant dose-dependent increases in the MDA production starting from 5 µM concentration (Figure 15b). Increases ranged from 0.98 folds ($p \leq 0.01$) to 1.64 folds ($p \leq 0.001$) respect to the control for 5 µM and 20 µM concentrations, respectively (Figure 15b). Regarding the individual treatment of 3-ADON the results show a significant increase in the MDA production at all concentrations as follows: 0.78 folds ($p \leq 0.01$), 1 fold ($p \leq 0.01$), and 1.32 folds ($p \leq 0.001$) at 1µM, 2µM, and at 4µM, respectively compared to the control (Figure 15c).

4.2.3.5. Lipid peroxidation assay of combined exposure to AA, PEN A, and 3-ADON

Results of LPO for combined exposure of AA, PEN A and 3-ADON are reported in Figure 15. The combination of [AA + PEN A] increased the MDA production in the range of 2.05 folds ($p \leq 0.01$) to 1.83 folds ($p \leq 0.01$) corresponding to [1250 + 5] µM and [5000 + 20] µM, respectively (Figure 15d). Regarding the [AA + 3-ADON] combination, there was an increase of the MDA production at all concentrations, ranging from 0.68 folds ($p \leq 0.01$) to 1.57 folds ($p \leq 0.001$) for [2500 + 2] µM and [5000 + 4] µM, respectively compared to the control (Figure 15e). Concerning the combination of [PEN A + 3-ADON] there was an increase at the highest concentrations tested of 2.63 folds at [10 + 2] µM ($p \leq 0.001$) and of 3.45 folds at [20 + 4] µM ($p \leq 0.001$) compared to the control (Figure 15f).

Lastly, the tertiary combination revealed a significant increase at all concentrations tested from 0.81 folds for [5000 + 20 + 4] µM ($p \leq 0.01$) to 1.21 folds for [1250 + 5 + 1] µM ($p \leq 0.01$) compared to the control (Figure 15g).

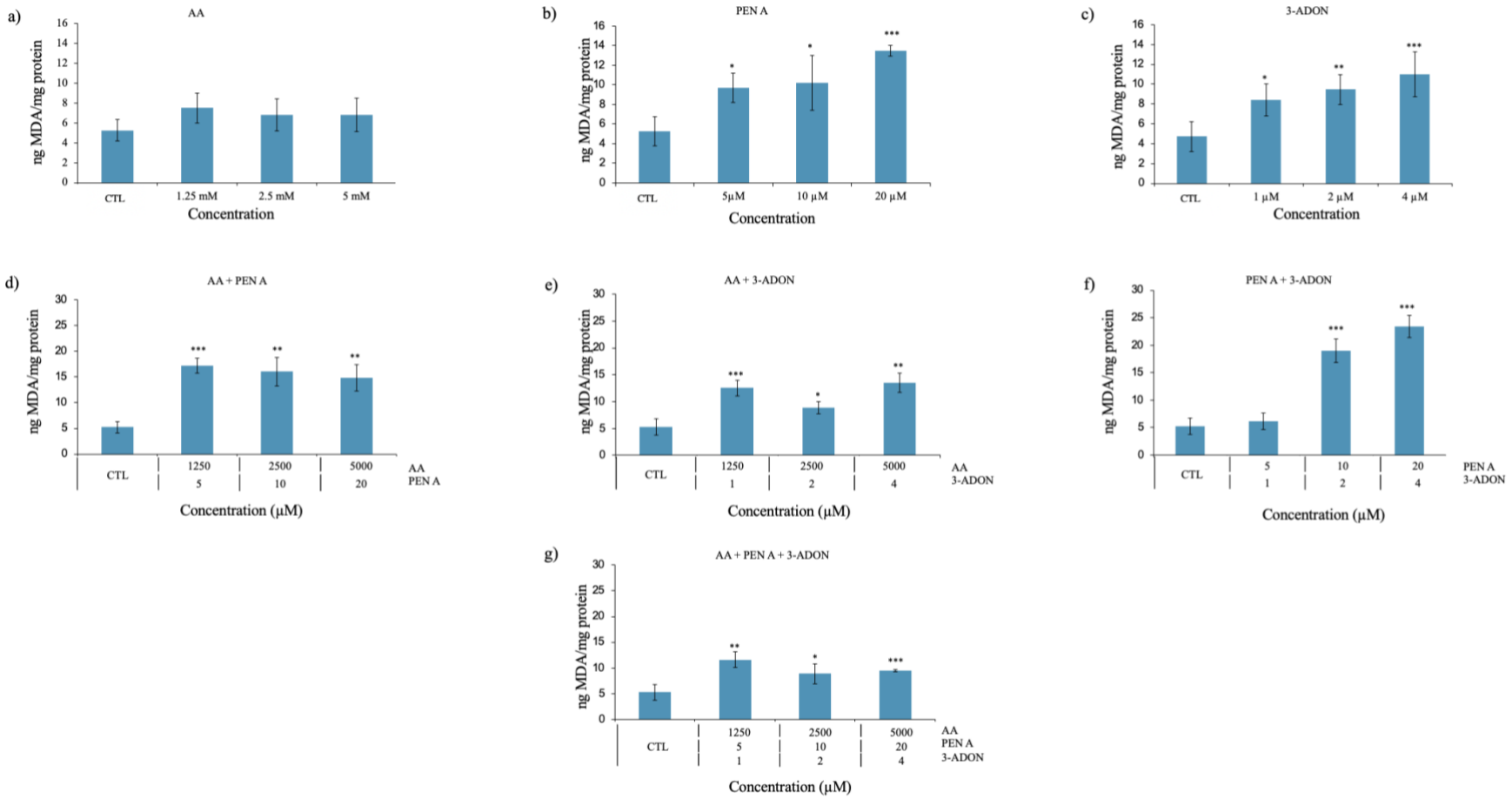


Figure 15. *Lipid peroxidation (LPO), measured as malondialdehyde (MDA) production, in SH-SY5Y cells incubated for 24 h with: a) AA at 1.25, 2.5, and 5 mM; b) Pen A at 5, 10, and 20 µM; c) 3-ADON at 1, 2, and 4 µM; d) AA + Pen A at 1.25, 2.5, and 5 mM combined with 5, 10, and 20 µM, respectively; and e) AA + 3-ADON at 1.25, 2.5, and 5 mM combined with 1, 2, and 4 µM, respectively.*

4.2.3.6. Glutathione determination of individual and combined exposure to AA, PEN A, and 3-ADON

Results of GSH, GSSG and GSH/GSSG ratio variation in SH-SY5Y cells was carried out as described in section 3.11. Results of GSH/GSSH ration have reported in Figure 18; while measurement of GSH and GSSG for individual and combination mixture of mycotoxins are collected in Figure 16 and 17, respectively. GSH levels for in individual treatment reached the highest significantly increased respect to the control for 2 µM 3-ADON (Figure16c) and the lowest level was reached for 5000 µM AA (Figure 16a); while in combined the values were below the control value (Figure 16a, 16b and 16c). GSSG levels for in individual treatment reached the highest significantly increased respect to the control for 4 µM 3-ADON (Figure 17c) and the lowest level was reached for 250 µM AA (Figure 17a); while in combined the values were significantly higher respect to the control ranging from 1 µg GSSG/mg protein to 1.4 µg GSSG/mg protein for [AA + PEN A] at [1250 + 5] µM and [PEN A + 3-ADON] at [5 + 1] µM, respectively (Figure 17d, 17e and 17f).

Regarding the GSH/GSSG ratio for individual treatment of SH-SY5Y cells the following results were obtained: i) for AA it significantly decreased in SH-SY5Y cells exposed to 2500 and 5000 µM by 15.1 % ($p \leq 0.01$) and 32.5 % ($p \leq 0.001$) respectively (Figure 18a); ii) for PEN A it significantly decreased by 21.3 % ($p \leq 0.01$), by 29.9% ($p \leq 0.001$) and by15.8% ($p \leq 0.05$) at 5 µM, 10 µM and 20 µM, respectively (Figure 18b); lastly, iii) for 3-ADON the GSH/GSSG ratio significantly decreased at 4 µM by 18.7 % ($p \leq 0.05$) and at 1 µM by 15.7 % ($p \leq 0.05$) compared to control (Figure 18c).

When analysing the results of GSH/GSSG ratio for binary treatment it was observed that: i) for [AA + PEN A] combination a decreased at all concentrations was observed, ranging from 13.9 % ($p \leq 0.05$) to 27.9 % ($p \leq 0.001$) for [1250 + 5] µM and [5000 + 20] µM, respectively respect to the control (Figure 18d); ii) for [AA + 3-ADON] treatment, the GSH/GSSG ratio was reduced in all cases ~ 30 % for all scenarios, respect to the control (Figure 18e); and iii) for [PEN A + 3-ADON] ratio was reduced in the range

from 26.6 % (p≤ 0.001) to 36.6 % *(p≤ 0.001)* for [5 + 1 +] µM and [20 + 4] µM, respectively respect to the control (Figure 18f).

With respect to the tertiary combination of AA + PEN A + 3-ADON, the GSH/GSSG ratio was significantly reduced at all concentrations compared to the control in all cases around ~ 30 % respect to the control (*p≤ 0.001*) (Figure 18g).

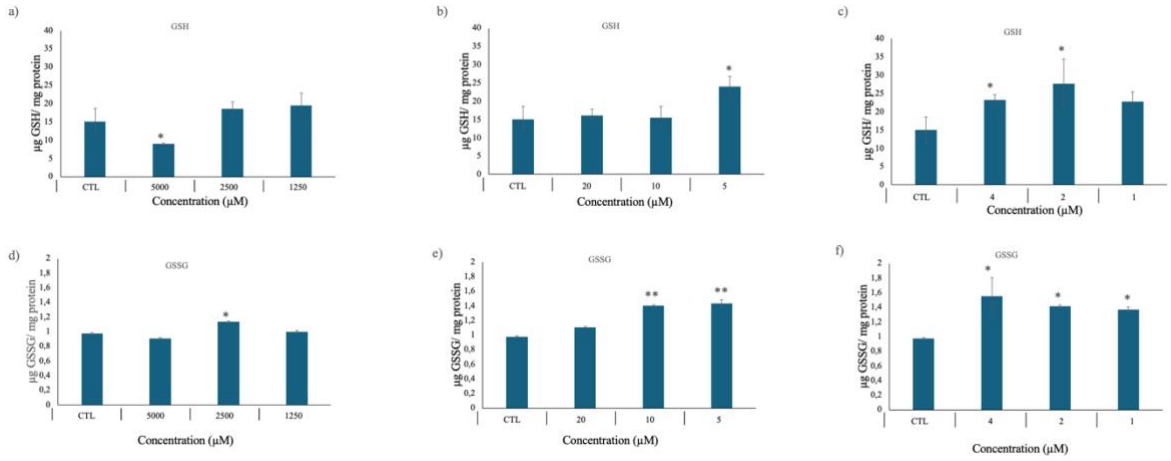


Figure 16. *Effect of a) AA at 1.25, 2.5, and 5 mM, b) PEN A at 5, 10, and 20 µM and c) 3-ADON at (1250 µM , 2500 µM, 5000µM) + (5 µM , 10 µM, 20 µM) + (1 µM, 2 µM, 4 µM) on GSH levels (a, b, and c) and GSSG levels (d, e, and f), after 24 h of exposure. Data are expressed as mean values ± SEM of two independent experiments with 4 replicates each. *p ≤ 0.05 indicates a significant difference respect to the fresh medium.*

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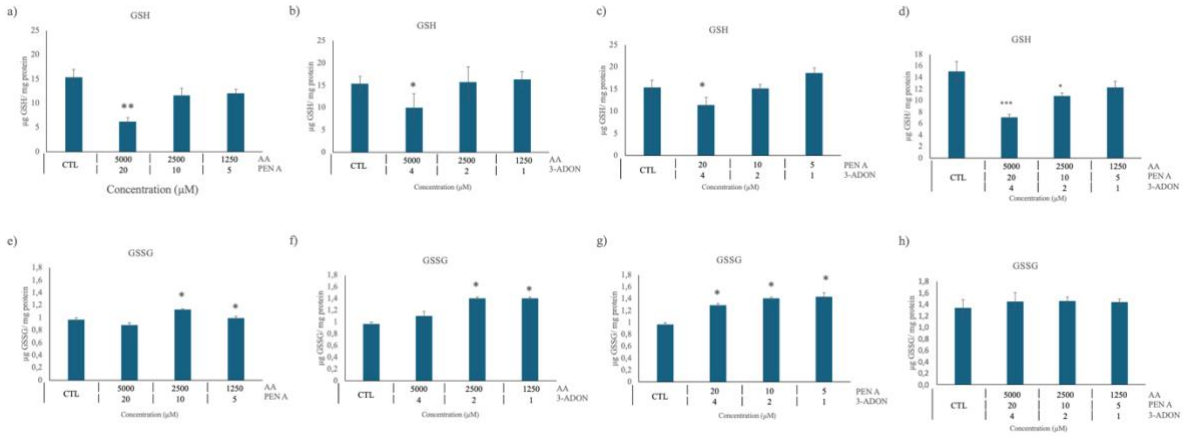

Figure 17. *Effect of AA + PEN A at 1 (1250 µM , 2500 µM, 5000µM) + (5 µM , 10 µM, 20 µM), e) AA + 3-ADON (1250 µM , 2500 µM, 5000µM) + (1 µM, 2 µM, 4 µM), f) PEN A + 3-ADON at (5 µM , 10 µM, 20 µM) + (1 µM, 2 µM, 4 µM), and g) AA + PEN A + 3-ADON at (1250 µM , 2500 µM, 5000µM) + (5 µM , 10 µM, 20 µM) + (1 µM, 2 µM, 4 µM) on GSH levels (a) and GSSG levels (b), after 24 h of exposure. Data are expressed as mean values ± SEM of two independent experiments with 4 replicates each. *p ≤ 0.05 indicates a significant difference respect to the fresh medium.*

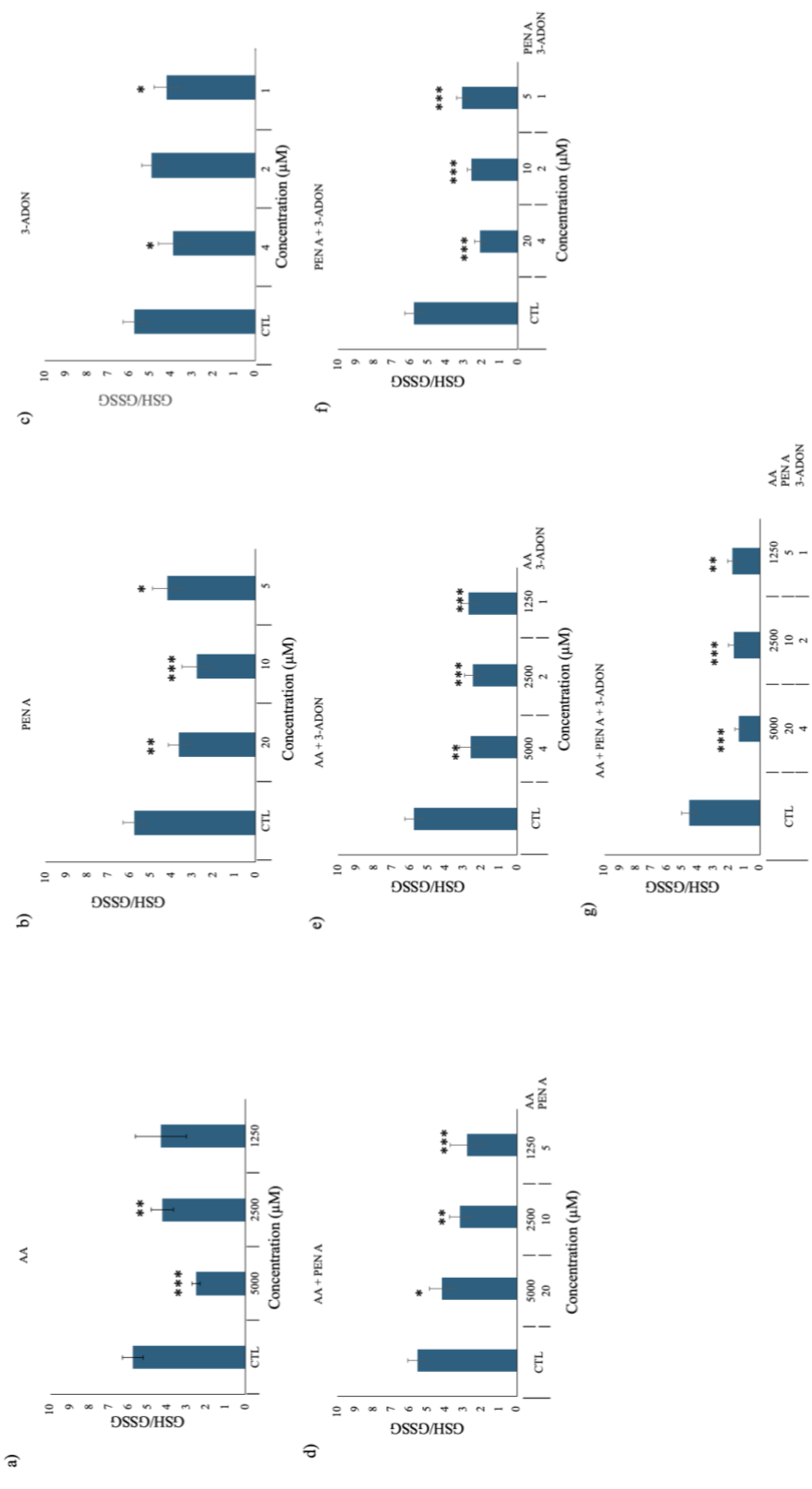

Figure 18. *Effect on the GSH/GSSG ratio in SH-SY5Y cells after 24 h of exposure to: a) AA at 1.25, 2.5, and 5 mM; b) Pen A at 5, 10, and 20 μM; c) 3-ADON at 1, 2, and 4 μM; d) AA + Pen A at 1.25, 2.5, and 5 mM combined with 5, 10, and 20 μM, respectively; e) AA + 3-ADON at 1.25, 2.5, and 5 mM combined with 1, 2, and 4 μM, respectively; f) Pen A + 3-ADON at 5, 10, and 20 μM combined with 1, 2, and 4 μM, respectively; and g) AA + Pen A + 3-ADON at 1.25, 2.5, and 5 mM + 5, 10, and 20 μM + 1, 2, and 4 μM, respectively. Data are expressed as mean ± SEM of two independent experiments with four replicates each. $p \leq 0.05$ indicates a significant difference compared to cells in fresh medium.*

4.2.4. Discussion

The negative impact of AA and PEN A in the nervous system of humans and animals has been previously reported (2014; Rundberget et al., 2010; Zhao, 2022). While for 3-ADON it has been reported negative impacts in different cell lines (Alassane-Kpembi et al., 2015; Juan-García et al., 2019a, 2016; Pinton et al., 2012) but never in a neuronal cells/system neither the combination of AA, PEN A, and 3-ADON. The present work reports for the first time the individual study of 3-ADON in SH-SY5Y cells as well as the AA, PEN A, and 3-ADON combined effect in human neuroblastoma SH-SY5Y cells in relation to time, mixture, and compound exposure. Therefore, the aim of this work was to stablish the impact of food contaminants individually and in combination in the oxidative stress response in human neuroblastoma, SH-SY5Y cells. In general terms, the present study reveals an increase in the LPO and a decrease in the GSH/GSSG ratio for the combinations where AA intervene and respect to AA individual exposure, confirming the oxidative stress response. Therefore, these findings suggest that the combined effect induced higher lipid peroxidation in SH-SY5Y cells and with higher toxic effect than the individual treatment of AA. Nevertheless, for this compound (AA) the effect in ROS production diminished, which could be attributed to the differing exposure times used in the two assays, and that the cocktail of mycotoxins had a more complex behavior, than the individual exposure. ROS production was assessed within 2 hours at sublethal concentrations, whereas LPO levels and GSH/GSSG ratio were measured after 24 hours of treatment.

In detail, in one hand the interactions between [AA + PEN A] previously reported by Bridgeman et al., 2023 state that each mycotoxin when tested individually presented a higher cytotoxic response than the combination of both (Bridgeman et al., 2023) similar tendency has been previously reported by other authors (Agahi et al., 2020c). Nevertheless, no studies have been found about the effects of 3-ADON in SH-SY5Y cells, neither when combined with AA and PEN A. On the other hand, several studies have stated the importance of studying the interactions between AA combined to other mycotoxin as well as the combination among mycotoxins (Bridgeman et al., 2023; Mitchell et al., 2024; Pyo et al., 2020; Su et al., 2023). When compared the 3-ADON

with the double and tertiary combination of AA and PEN A, it can be stated that the tertiary combination was the most cytotoxic combination, followed by the double combination of [AA + 3-ADON], and then [PEN A + 3-ADON], the less cytotoxic was 3-ADON individually.

Regarding the ROS results obtained for AA individually several authors have stablished that AA induces oxidative stress by increasing the ROS production and decreasing the GSH (Pan et al., 2018; Zhao et al., 2017) in line with our results. Nevertheless, other authors (Johansson et al., 2024) have stablished that after 9 days of exposure of AA at non-cytotoxic concentrations (0.33 μM and 10 μM) did neither decrease the level of GSH or total glutathione levels (GSH + GSSH), nor increased ROS production for the same cell line as that reported in this study. ROS results for PEN A, were similar to those obtained in other neuronal cell lines (cerebellar granule neuron and human neutrophils), revealing increases at similar concentrations as those tested by us (10 μM, 25 μM and 50 μM) (Berntsen et al., 2013). While for 3-ADON, it has been reported no increase of ROS in HepG2 cells at a concentrations bellow those tested here, which it does coincides with the results reported in here (Juan-García et al., 2019a).

ROS generation in binary combinations [AA + PEN A], [PEN A + 3-ADON] and their tertiary, if compared to individual treatments, there was always a decrease of the ROS production. Therefore, it seems like the individual treatments induced more ROS than the combination, a similar tendency was obtained by other authors when exposing SH-SY5Y and other cell lines to mixtures of mycotoxins; as it is the case of Fumonisin B1 (FB1) and Ochratoxin A (OTA) in SH-SY5Y (Penalva-Olcina et al., 2023), beauvericin (BEA) and OTA in HepG2 (Juan-García et al., 2020), or for zearalenone (ZEA) and aflatoxin B1 (AFB1) in cell porcine kidney cells suggesting an antagonistic effect in oxidative damage (Lei et al., 2013). No studies have been found for the triple combination of AA + PEN A + 3-ADON in the oxidative stress response. Nevertheless, other studies, in the same cell line, carried out tertiary combination of different mycotoxins, such as α-ZEL + β-ZEL + BEA, where a decrease in ROS generation was

observed (Agahi, et al., 2020), similar to our results, where individual exposure induced more ROS generation than the tertiary combination.

Regarding the effect on the LPO an increase of MDA in SH-SY5Y cells exposed to AA was consistent with the result in bibliography in which it was state an increase in the LPO marker levels in different tissues and cell lines upon AA exposure (Allam et al., 2013; Yousef and El-Demerdash, 2006). Furthermore, it has been suggested that the enhancement of LPO is a consequence of glutathione depletion to certain critical levels (Srivastava et al., 1983). The increase of MDA in PEN A and 3-ADON is aligned with the previous results obtained for other mycotoxins in different cell lines (Mézes et al., 2021). On the contrary, there was no increase in the MDA production after 24 h in HepG2 cells (Juan-García et al., 2019a), this difference may be due to the different concentrations tested or the different cell line. In binary combinations [AA + PEN A], [AA + 3-ADON] and [PEN A + 3-ADON], it was observed in general terms an increase of MDA if compared to each compound tested individually in line with other studies that stated that the mixture of mycotoxins increased the lipid peroxidation in Laying Hens (Kulcsár et al., 2024).

For the tertiary exposure [AA + PEN A + 3-ADON] levels of MDA compared to the individual treatment where different according to the compounds as it increased at all concentrations if compared to AA, opposite to what is was observed for PEN A as a decrease was observed; while for 3-ADON an oscillation was observed according to the concentration tested (increase and decrease). It can be set that the combination showed an additive or synergetic tendency, similarly to a study carried out in Caco-2 cells for other mycotoxins where the binary mixtures of deoxynivalenol (DON) with ZEA or fumonisin B1 (FB1) increased MDA production in a synergistic way whereas mixture of [ZEA + FB1] and mixture of all three mycotoxins show additive effects after 24h of exposure (Kouadio et al., 2007).Similarly, BEA and OTA increased the MDA production individually and in combination in HepG2 cells(Juan-García et al., 2020). Also, in HepG2, the combinations of DON + 3-ADON and 15-ADON revealed an

increase in MDA production in mixtures or in combination of all three mycotoxins (Juan-García et al., 2019).

AA is oxidized to glycidamide, a reactive epoxide, and undergoes conjugation with reduced glutathione (GSH) (Ghanayem et al., 2005). DNA adducts from glycidamide have been reported following AA administration (Doerge et al., 2005; Dybing and Sanner, 2003). Combined exposures to AA, PEN A, and 3-ADON caused a more pronounced reduction in the GSH/GSSG ratio across various concentrations compared to individual treatments. The tertiary mixture showed the most significant reduction, especially at high and low concentrations. These findings suggest that the combined exposure decrease of intracellular GSH at the highest concentrations and a significant increase of GSSG levels at lowest concentrations in SH-SY5Y cells as a response to oxidative damage. Similar tendency was obtained for different mycotoxins combinations in porcine kidney cells (Klarić et al., 2007). On the contrary, in the same cell line, but different mycotoxins (Beauvericin (BEA), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL)), there was an increase in the GSH/GSSG ratio for all the treatments individually and in combination (Agahi, et al., 2020). While for the same mycotoxins (α-ZEL + β-ZEL + BEA) the GPx activity obtained a higher increase in individual exposure than in combinations (Agahi, et al., 2020).

The results have significant implications for understanding how mycotoxin interactions contribute to oxidative stress and cellular damage in neuronal systems, as well as drawing attention to the need for regulatory considerations regarding combined mycotoxin exposure in food.

4.2.5. Conclusion

In conclusion this work provides new insights into the toxic effects of AA, PEN A, and 3-ADON, both individually and in combination, on oxidative stress in human neuroblastoma SH-SY5Y cells. While the negative impacts of AA and PEN A on the nervous system have been previously documented, this is the first study to investigate

the individual effects of 3-ADON in SH-SY5Y cells and the combined impact of these three food contaminants. The findings confirm that the combined exposures induced higher lipid peroxidation and reduced GSH/GSSG ratios, highlighting the synergistic or additive effects of these mycotoxins might have. These findings suggest that oxidative stress plays a central role in individual and combined AA, PEN A and 3-ADON induced cytotoxicity. This study emphasizes the importance of exploring such combinations, as the mixtures seemed to be more toxic than the individual contaminants. Further studies are still needed to investigate other intracellular mechanisms of action potentially involved in the toxicity induced by AA PEN A and 3-ADON individual and combined exposure.

CHAPTER III: 4.3. Evaluating the genotoxicity and mutagenicity of food contaminants: acrylamide, penitrem A, and 3-acetyldeoxynivalenol in individual and combined exposure *in vitro*.

4.3.1. Introduction

Understanding the genotoxicity of food contaminants is critical, particularly when assessing compounds formed during food processing or produced naturally by fungi. Acrylamide (AA), penitrem A (PEN A), and 3-acetyldeoxynivalenol (3-ADON) are three such contaminants that pose notable health concerns due to their genotoxic and toxic properties (Bridgeman et al., 2023; Han et al., 2014; Riboldi et al., 2014). Recent research has increasingly focused on their effects at the neuronal level, with SH-SY5Y neuroblastoma cells emerging as a relevant in vitro model (Bridgeman et al., 2024; Lopez-Suarez et al., 2022).

In addition to neuronal genotoxicity, mutagenicity testing is essential and typically requires bacterial models, such as those used in the Ames test (OECD, 2020). By integrating both cellular and bacterial systems, a more comprehensive understanding of the toxicological impact of these compounds can be achieved. This study applies both the Ames and micronucleus (MN) assays to evaluate mutagenic and genotoxic responses to AA, PEN A, and 3-ADON, individually and in combination.

Acrylamide, widely used in industry, was unexpectedly discovered in food in 2002, raising public concern due to its classification as a probable human carcinogen (Capuano and Fogliano, 2011). It forms during high-temperature cooking of carbohydrate-rich foods, such as frying or baking, and is commonly found in items like potatoes, bread, and coffee (Sebastià et al., 2023). After ingestion, AA is metabolized by CYP2E1 into glycidamide, a reactive epoxide capable of forming DNA adducts, thus contributing to genotoxicity through chromosomal aberrations and MN induction (Chen et al., 2022; Pifferi et al., 2024; Benford et al., 2022).

Beyond process contaminants, exposure to mycotoxins such as PEN A and 3-ADON through contaminated food and feed is also widespread. These natural toxins can co-occur with AA in cereal-based products like bread, biscuits, and processed grains, making the study of combined exposure highly relevant for realistic food safety risk

assessment. PEN A, a potent neurotoxin produced by *Penicillium crustosum*, is known to impair neurotransmission by inhibiting BK channels and disrupting GABAergic signaling (Berntsen et al., 2017; Eriksen et al., 2013). However, its genotoxicity has not yet been assessed using MN assays, representing a significant knowledge gap addressed in this study.

3-ADON, a type B trichothecene mycotoxin derived from deoxynivalenol (DON), is commonly found in cereals and grains (Jurado et al., 2005). DON is thermally stable, and the toxicity of its degradation products remains uncertain (Kabak, 2009). Although not classified as carcinogenic, both DON and 3-ADON have shown genotoxic potential, including chromosomal damage, in various in vitro models (Juan-García et al., 2018; Singh et al., 2015).

Genotoxicity testing is essential to detect substances that may cause mutations, chromosomal aberrations, or DNA damage (Ren et al., 2017; Hartwig et al., 2020). In regulatory toxicology, two core assays are widely accepted: the MN test, which detects chromosomal damage, and the Ames test, which assesses point mutations (EFSA, 2011; Kirkland et al., 2011). Combined, these tests provide a robust strategy for identifying rodent carcinogens and in vivo genotoxins. Their complementary nature—detecting gene mutations and structural/numerical chromosome alterations—makes them a cornerstone of in vitro genotoxicity assessment. The MN test, validated and standardized by the OECD (2010), is widely used due to its reliability and versatility, while the Ames test remains a standard for screening mutagenic potential (Thomas et al., 2023).

Importantly, real-life dietary exposure involves complex mixtures rather than isolated compounds, yet most toxicological studies have focused on single-substance exposure. The novelty of this work lies in its assessment of combined exposure to AA, PEN A, and 3-ADON, as well as in the first-time investigation of PEN A-induced micronucleus formation. Such an approach better reflects realistic risk scenarios and helps fill critical gaps in regulatory and scientific knowledge.

Therefore, the aim of this study is to evaluate the genotoxic and mutagenic effects of AA, PEN A, and 3-ADON, both individually and in combination, using the MN assay in SH-SY5Y cells and the Ames test across multiple bacterial strains.

4.3.2. Material and methods

2.1. Reagents (described in section 3.1.)

4.2. Cell culture (described in section 3.2.)

4.3. Mycotoxin exposure (described in section 3.5.)

2.4. Micronucleus assay by flow cytometry (described in section 3.12.)

2.5. Bacterial reverse mutation test or Ames test (described in section 3.13.)

2.6. Statistical analysis (described in section 3.14.)

4.3.3. Results

4.3.3.1. Micronucleus induction in individual and combined exposure to AA, PEN A and 3-ADON

The results obtained after the exposure of AA, PEN A and 3-ADON, in the MN frequencies on SH-SY5Y cells are reported in Figure 17. All the three individual treatments increased the effect on MN frequency significantly. Specifically, for AA, there was a dose-dependent increase of 6.4 % ($p \leq 0.01$), 11.7 8 % ($p \leq 0.01$), and 28.32 % ($p \leq 0.001$), for 625, 1250, and 2500 µM, respectively (Figure 17a). Regarding PEN A, there was also an increase at all concentrations of 27.88 % ($p \leq 0.001$), 14.17 % ($p \leq 0.001$), and 22.98 % ($p \leq 0.001$) for 2.5, 5, and 10 µM, respectively (Figure 17b). For the 3-ADON there was also a dose-dependent increasing tendency, of 15.12 % ($p \leq 0.05$), 18.32 % ($p \leq 0.05$), and 24.63 % ($p \leq 0.05$) for 1, 2, and 4 µM, respectively (Figure 15c).

Regarding the combined treatments collected in Figure 18, it can be observed that [AA + PEN A] increased the MN frequencies at the lowest concentrations, up to 26.57 % ($p \leq 0.01$) and 18.36 % ($p \leq 0.05$) for [625 + 2.5] µM, and [1250 + 5] µM, respectively (Figure 18a). While [AA + 3-ADON] exhibit an increase at all concentrations, of 18,05 % ($p \leq 0.05$), 25.05 % ($p \leq 0.01$), and 26.31 % ($p \leq 0.05$), for [625 + 1] µM, [1250 + 2] µM and [2500 and 4] µM, respectively (Figure 18b). Concerning the [PEN A + 3-ADON] combination, there was an increase at the highest concentrations of 7.04 % ($p \leq 0.05$) and 30.42 % ($p \leq 0.01$), for [5 + 2] µM and [10 + 4] µM, respectively (Figure 18c). Finally, for the tertiary combination there was also a significative increase for the highest concentrations of 6.19 % ($p \leq 0.01$) and 34.16 % ($p \leq 0.001$), for [1250 + 5 + 2] µM and [2500 + 10 + 4] µM, respectively (Figure 18d).

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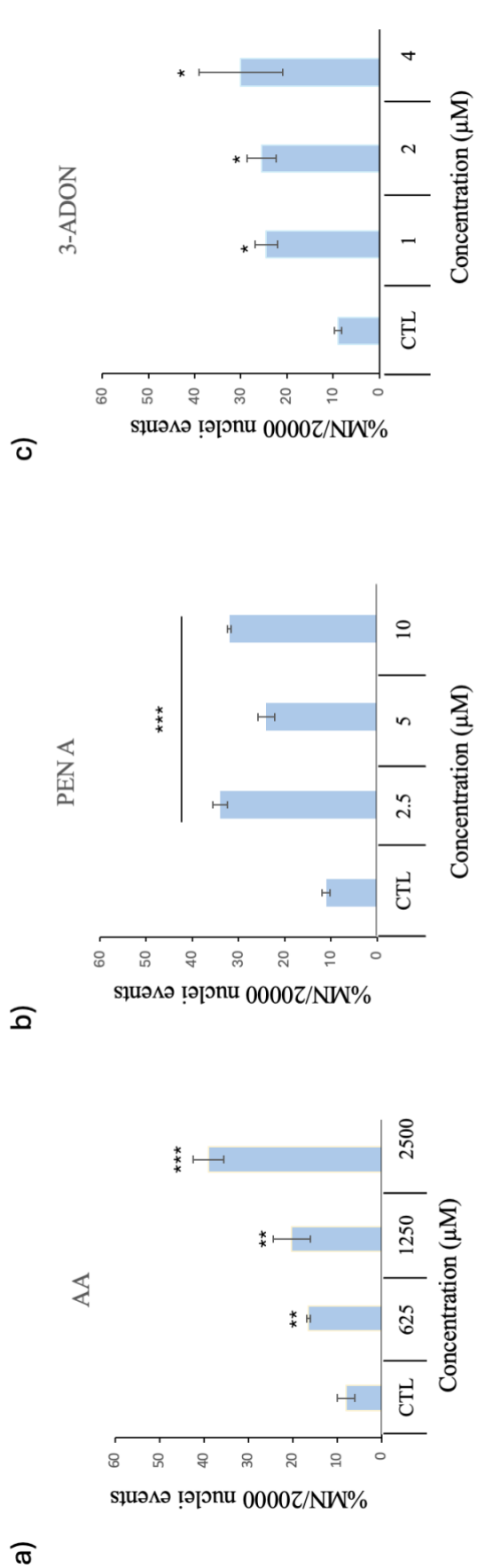


Figure 19. Induction of micronuclei in SH-SY5Y cells treated individually by (a) AA, (b) PEN A and (c) 3-ADON, at several concentrations for 48 h. Results are expressed as percentage of MN per 20000 cells ±SEM (n = 3). $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.000$ (***), significantly different from the control.

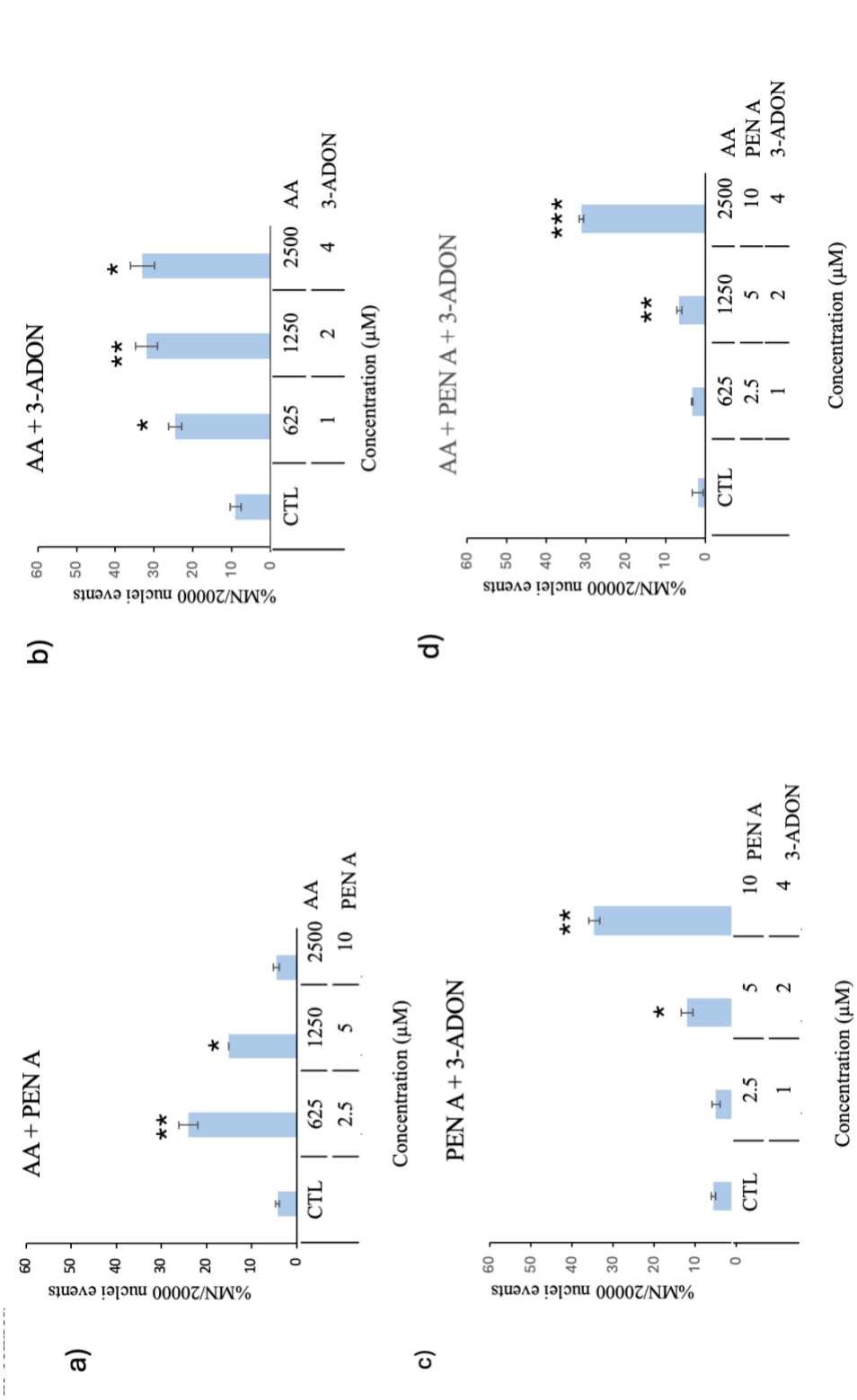


Figure 20. Induction of micronuclei in SH-SY5Y cells treated in binary combination by (a) AA + PEN A, (b) AA + 3-ADON and (c) PEN A + 3-ADON, and tertiary combination of (d) AA + PEN A + 3-ADON at several concentrations for 48 h. Results are expressed as percentage of MN per 20000 cells ±SEM (n = 3). $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.000$ (***), significantly different from the control.

4.3.3.2. Ames test individual and combined exposure to AA, PEN A and 3-ADON

The results of the Ames test (with/without exogenous metabolic activation) performed with AA, PEN A and 3-ADON, are presented in Table 19. As mentioned in section 2.5 a result was considered positive if the number of revertant colonies was two-fold higher than the number of revertant colonies in the solvent control plates for TA98, TA100, and *E.coli* WP2 strains, and three-fold for TA1535 and TA1537 and if a dose-dependent response was observed.

AA, PEN A, and 3-ADON were no cytotoxic for the bacteria at the highest concentrations assayed, with or without exogenous activation system. In this study, none of the individual or combined results of the Ames test (+S9 or −S9) exceeded twice the value of the solvent (DMSO) control except for AA at all concentrations in TA 1537 with S9 with concentration–effect profile and 3-ADON at the lowest concentrations (1 and 2 μM) in TA 1537 without S9 without concentration–effect profile. Therefore, the Ames test did not show any genotoxic potential of AA, PEN A and 3-ADON compared to the respective positive controls (Table 19,20,21). Similarly to the individual treatments, the results of the Ames test (with/without exogenous metabolic activation) performed with the double and tertiary combinations are presented in Table 20 and 21. [AA + PEN A], [AA + 3-ADON], [PEN A + 3-ADON] and [AA + PEN A + 3-ADON], were no cytotoxic for the bacteria at the highest concentrations assayed, with or without exogenous activation system except for the highest concentration of [PEN A + 3-ADON] [10 + 4] μM in TA100 without S9.

Table 19. Results in the bacterial reverse mutation assay after the exposure of AA at 625 µM, 1250 µM and 2500 µM, PEN A at 2.5 µM, 5 µM and 10 µM, and 3-ADON at 1 µM, 2 µM and 4 µM to Salmonella typhimurium and Escherichia coli WP2 strains, in the absence or presence of S9 fraction in the growth medium.

	Concentration (µM)	TA98		TA100		TA1535		TA1537		WP2	
		-S9	+S9	-S9	+S9	-S9	+S91	-S9	+S9	-S9	+S9
	Negative control[a]	4	2	28	30	2	6	2	3	23	32
AA	**625**	2	5	26	31	1	1	1	69	23	29
	1250	3	5	27	32	3	5	1	71	20	43
	2500	4	4	30	31	1	1	2	124	29	43
PEN A	**2.5**	5	3	32	20	2	3	6	1	23	33
	5	3	5	21	21	4	1	4	1	20	33
	10	3	4	17	24	3	1	4	3	26	45
3-ADON	**1**	3	2	18	17	1	2	26	5	21	29
	2	2	3	31	14	2	3	7	2	25	32
	4	3	4	29	9	0	1	5	2	24	37
	Positive control	34[b]	20[c]	270[d]	512[c]	274[d]	58[c]	40[e]	154[c]	692[f]	144[c]

[a] 1% DMSO; [b] 2-Nitrofluorene (2-NF); [c] 2-Aminoanthracene (2-AA); [d] AzN_3; [e] 9-aminoacridine (9-AC); [f] 4-Oxide of nitroquinoline
*Cytotoxicity

Table 20. Results in the bacterial reverse mutation assay after the exposure of AA + PEN A at [625 + 2.5] µM, [1250 + 5] µM and [2500 + 10] µM, AA + 3-ADON at [625 + 1] µM, [1250 + 2] µM and [2500 + 4] µM, and PEN A + 3-ADON at [2.5 + 1] µM, [5 + 2] µM and [10 + 4] µM to Salmonella typhimurium and Escherichia coli WP2 strains, in the absence or presence of S9 fraction in the growth medium.

	Concentration	TA98		TA100		TA1535		TA1537		WP2	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
	Negative control[a]	4	2	28	30	2	6	2	3	23	32
AA + PEN A	**[625 + 2.5] µM**	4	4	29	29	3	7	1	2	14	41
	[1250 + 5] µM	3	5	33	27	2	4	1	1	14	35
	[2500 + 10] µM	2	3	30	24	2	9	0	1	14	38
AA + 3-ADON	**[625 + 1] µM**	2	5	28	24	3	6	1	3	12	29
	[1250 + 2] µM	5	7	29	28	1	5	2	3	14	40
	[2500 + 4] µM	3	7	29	26	1	6	1	3	20	32
PEN A + 3-ADON	**[2.5 + 1] µM**	2	5	23	33	1	4	5	2	15	20
	[5 + 2] µM	2	5	19	29	2	7	4	1	16	32
	[10 + 4] µM	4	4	-*	26	1	7	5	2	18	28
AA + PEN A + 3-ADON	**[625 + 2.5 + 1] µM**	3	7	31	29	1	7	1	2	20	26
	[1250 + 5 + 2] µM	4	5	30	28	3	7	1	2	20	35
	[2500 + 10 + 4] µM	5	4	28	31	1	7	1	1	21	36
	Positive control	34[b]	20[c]	720[d]	512[c]	69[d]	71[c]	40[e]	42[c]	855[f]	142[c]

[a] 1% DMSO; [b] 2-Nitrofluorene (2-NF); [c] 2-Aminoanthracene (2-AA); [d] AzN_3; [e] 9-aminoacridine (9-AC); [f] 4-Oxide of nitroquinoline
*Cytotoxicity

Table 21. Results in the bacterial reverse mutation assay after the exposure of AA + PEN A + 3-ADON at [625 + 2.5 + 1] µM, [1250 + 5 + 2] µM and [2500 + 10 + 4] µM, to *Salmonella typhimurium* and *Escherichia coli* WP2 strains, in the absence or presence of S9 fraction in the growth medium.

	Concentration	TA98		TA100		TA1535		TA1537		WP2	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
	Negative control[a]	3	3	26	15	2	6	1	2	23	32
	[625 + 2.5 + 1] µM	3	7	31	29	1	7	1	2	20	26
AA + PEN A + 3-ADON	**[1250 + 5 + 2] µM**	4	5	30	28	3	7	1	2	20	35
	[2500 + 10 + 4] µM	5	4	28	31	1	7	1	1	21	36
	Positive control	32[b]	20[c]	191[d]	232[c]	69[d]	71[c]	40[e]	42[c]	855[f]	142[c]

[a] 1% DMSO; [b] 2-Nitrofluorene (2-NF); [c] 2-Aminoanthracene (2-AA); [d] AzN_3; [e] 9-aminoacridine (9-AC); [f] 4-Oxide of nitroquinoline
*Cytotoxicity

4.3.4. Discussion

In this study, in order to assess the genotoxic ability of potential food contaminants, we used two short-term *in vitro* assays directed at different genotoxic endpoints in different phylogenetic systems: i) the MN assay, a commonly used technique to evaluate DNA damage at a chromosome level; and, ii) the Ames test, a well-accepted tool for the assessment of the mutagenicity of chemicals. The combination of both tests fulfils the basic requirements to cover the three genetic endpoints to stablish if a substance is related to genotoxic or mutagenic effects; the *in vitro* MN test covers structural chromosome aberrations while the Ames test (bacterial reverse mutation assay) covers gene mutations (Benford et al., 2022). These two tests are effective for identifying most potential genotoxic substances. Adding other *in vitro* tests to them would notably decrease specificity without providing a meaningful improvement in sensitivity (Benford et al., 2022).

Regarding the MN assay conducted according to the methods described in TG OECD 487 (OECD/OCDE 487, 2023) and previously used in mycotoxins studies by Juan García *et al.,* (Juan-García et al., 2019, 2018), we observed an increase in the MN frequency at all the concentrations assayed for AA, PEN A and 3-ADON, as well as for the combinations. Regarding AA, similar results were obtained in human lymphocytes by Zamani et al., (2018) with a significant increase in frequency of MN at concentrations of 50 and 100 mM. In another study, conducted in mouse bone marrow cells, AA significantly increased the frequency of micronucleated cells in exposed groups compared to the negative control, supporting again that AA may induce genotoxicity (Algarni, 2018). Further investigation into AA has revealed species-specific differences *in vivo*. For instance, a dose-dependent increase in MN formation was observed in mice but not in rats, potentially due to higher glycidamide levels in mice (Hobbs et al., 2016). Additionally, AA caused 10- and 20-fold increases in MN frequencies in morphologically normal and abnormal mouse embryos, respectively, with 41 and 93 MN per 1,000 cells (Titenko-Holland et al., 1998). These findings are in line with the trend observed in our results, albeit in SH-SY5Y cells.

Regarding genotoxicity induced by mycotoxin exposure, a wide range of toxins and cell lines have been studied. According, MN formation was assessed in HepG2 cells, bone marrow polychromatophilic erythrocytes, and neuroblastoma SH-SY5Y cells, for the following mycotoxins: 15-acetyldeoxinyvalenol (15-ADON), aflatoxin B1 (AFB1), beauvericin (BEA), deoxynivalenol (DON), ochratoxin A (OTA), T-2 toxin, sterigmatocystin (STE) and patulin (PAT) ((Juan-García et al., 2019, 2018; Šmerák et al., 2001; Taroncher et al., 2024; Zingales et al., 2021) as well as in bone marrow cells, rabbit kidney cells, and human lymphocytes for fumonisin B1 (FB1) and citrinin (CTN) (Dönmez-Altuntas et al., 2007; Rumora et al., 2002; Song et al., 2014; Zhou et al., 2022). In detail, Juan García et al. (2018) stated that at 3 µM of 3-ADON, an increase of 13.1 % of MN was obtained compared to control; furthermore, at a range of 0.6 to 4,8 µM for DON, and of 0.2 to 1.5 µM for both, 3-ADON and 15-ADON the binary combinations of [DON + 3-ADON] and [DON + 15-ADON] also increased MN frequency in the range from 12.0 % to 23.8 % even at the lowest concentrations tested; while for [3-ADON + 15-ADON] the increase was more noticeable; reaching values of 43.8 % (Juan-García et al., 2018). On the other hand, for tertiary combinations [DON + 3-ADON + 15-ADON], all concentrations assayed revealed increases in MN frequencies from 31.2 % to 46.7 % with (Juan-García et al., 2018), being closer to those reported in this study for SH-SY5Y cells. Also, similar outcomes (an increase of 12.1 %) was obtained for T-2 toxin in HepG2 cells, at the highest concentration assayed (60 nM) (Taroncher et al., 2024). Coinciding with our cell line, there is only one study in which genotoxicity and MN induction have been investigated with STE mycotoxin, obtaining an increase 4 % with (Zingales et al., 2021). This tendency is consistent with those reported in our work, in which a clear increase in MN formation was observed in all individual treatments. Moreover, PAT significantly increased MN frequency by 35% in human hepatoma HepG2 cells at the highest tested concentration (0.75 µM) (Zhou et al., 2009) and also induced chromosomal aberrations in bone marrow cells (Song et al., 2014). Similarly, FB1 exposure led to a concentration- and time-dependent increase in MN frequency in rabbit kidney RK13 cells, ranging from 24 to 49 per 1,000 cells (Rumora et al., 2002). Additionally, CTN induced a significant, concentration-dependent increase in MN frequency, ranging from 2.07% to 4.73% at a concentration

of 10 to 100 µM, in human lymphocytes (Dönmez-Altuntas et al., 2007). While studies of BEA and OTA have revealed increases of 14.2 % and 12 % for BEA (1.25 µM) and OTA (25 µM), respectively in HepG2 cells; whereas combined ([BEA + OTA]) the increase in the MN frequency ranged from 24 % to 28.3 % compared to the control (Juan-García et al., 2019), all this cases similarly to our results. Among mycotoxins, AFB1 is undoubtedly the most genotoxic and although far from our results, indeed, AFB1 can promote in HepG2 an increase of 80% in MN formation compared to the control (at 9.6 µM AFB_1) (Singto et al., 2020) respect to the control.

Moving to combined exposure, the MN study of AFB1 jointly OTA has been carried out in bone marrow cells (Corcuera et al., 2015). For AFB1, MN induction was significantly high (up to 90 times the control levels), while no effect was reported for OTA (Corcuera et al., 2015). In that same study for combined treatment ([AFB1 + OTA]) a noticeable MN induction was reached (69 times the control levels) although it was lower than when AFB1 was individually tested, indicating a possible antagonist effect (Corcuera et al., 2015). Similar results were observed in our study, wherein the addition of AA to mycotoxins reduced MN frequency, suggesting a possible interference of AA in mycotoxins induced genotoxicity. One step further, in an animal model, it was found a significantly higher frequency of MN upon exposure to [T-2 toxin + AFB1] in comparison with the effect of AB1 alone (Šmerák et al., 2001). These outcomes align with our findings in which [PEN A + 3-ADON] showed an additive effect on MN promotion. The reason of the effects observed in combinations treatment in our study can be associated to some implications of AA, 3-ADOn or PEN-A in chromosome lagging that makes to have a inconsistency profile according to the doses and exposure time tested. Nevertheless, further investigations are needed to confirm such mechanism trigger by these contaminants.

Altogether, AA, PEN A, and 3-ADON, in both individual and combined treatments, demonstrated MN induction levels comparable to or exceeding those reported for other mycotoxins in various models, highlighting their potent genotoxic potential. These

findings emphasize the importance of evaluating food contaminants both individually and in combination to better understand their cumulative effects.

Although the mutagenicity of AA, PEN A, and 3-ADON has been previously studied (Bull et al., 1984; Eisenbrand, 2020; Hashimoto and Tanii, 1985; Sabater-Vilar et al., 2003; Wehner et al., 1978) no studies have evaluated their combined effects. Our negative mutagenic results align with earlier findings, which consistently demonstrated In particular, for AA previous studies reported no mutagenicity in *Salmonella* strains TA1535, TA1537, TA1538, TA98, and TA100, both in the presence and absence of metabolic activation (Bull et al., 1984; Hashimoto and Tanii, 1985). However, Crudo et al. (2023) observed a slight mutagenic effect for AA at 50 mM (10 times higher than concentrations used in our study) in TA100 in the presence of S9 activation, we obtained a similar tendency in TA1537 with metabolic activation, probably because is metabolized by cytochrome P450 2E1 to its reactive metabolite glycidamide (Hölzl-Armstrong et al., 2020). Although differences in strains and methodologies exist (such as the use of *E. coli* instead of *Salmonella* in this study) our findings are consistent with the literature. Regarding PEN A, using the Ames test with *Salmonella* strains (TA1535, TA1537, TA1538, TA98, and TA100) with and without metabolic activation, Sabater-Vilar et al. (2003) reported no mutagenic effects, consistent with our findings using *E. coli* WP2. Similarly, mutagenicity assays for 3-ADON at 5 times higher concentrations in the same *Salmonella* strains than ours, showed no mutagenic activity, aligning with our work, despite differences in the bacterial systems used (Wehner et al., 1978) no mutagenic potential for these substances individually, with or without S9 activation, across various strains.

Regarding the combination scenarios tested, it was not obtained mutagenicity at any combination or concentration tested. These findings could be related to the concentration tested, which mimics a wide range of real scenarios of exposure but are insufficient to see any mutagenic impacts. Similarly to our results, Ehrlich et al., (2002) obtained a MN positive induction in a dose-dependent manner for FB1, and an absence of revertant colonies in the Ames test under all test conditions for the TA98, TA100,

TA102, TA1535, 1537 stains. Regarding combinations of AA and mycotoxins, similar results were obtained in the study of mutagenicity and genotoxicity from AA with the *Alternaria* mycotoxin alternariol (AOH). The combination did not show synergistic effects during the combined treatments in TA98 and TA100 at similar concentrations of AA and 0.1 and 10 µM of AOH. However, it did show slight mutagenic effects, both in the presence and absence of the S9 fraction, at a concentration 10 times higher than ours (50 µM + 0.5/50 mM) of [AOH + AA] (Crudo et al., 2023).

Concerning the mycotoxins combinations, to our knowledge just trichothecenes and AFB1 mixtures have been carried out. For T-2 toxin and vomitoxin it was not showing mutagenic activity at the concentrations assayed 60 nM and 0.43 µM for T2 and 5.4µM for vomitoxin (Šmerák et al., 2001; Taroncher et al., 2024), similar to our work. However, in combination with AFB_1, authors showed a mutagenic effect significantly greater than AFB_1 alone in the Ames test (in strain TA98 at all concentrations) (Šmerák et al., 2001). In this case, the most mutagenic mixture was [AFB1 + T-2 + vomitoxin] (Šmerák et al., 2001). The dissonance of results is probably due to the fact that they used different mycotoxins than ours, specially AFB_1 that is genotoxic and mutagenic with metabolic activation, as previously stated in literature (Aydin and Rencüzoğullari, 2019). While the mutagenic effects of AFB_1 have been extensively studied (Šmerák et al., 2001) and linked to its metabolite 8,9-epoxide-aflatoxin B1, inducing chromosomal aberrations, micronuclei formation, and other genotoxic outcomes, the mutagenic potential of the mycotoxins studied in this work remains unknown.

The absence of mutagenic response in the bacterial revers mutation assay observed for AA, PEN A, 3-ADON and its combination is consistent with our results. Nevertheless, based on our findings there is no available data in the literature regarding the mutagenic activity of our mycotoxin combinations or with AA as well as for our stains. Therefore, more studies are needed in order to better understand the mechanism of action underlying the genotoxicity of these compounds. Highlighting the need for further research, as combined mycotoxins with food processed contaminants could be a genuine health risk to the human population.

4.3.5. Conclusions

This work studies the mutagenicity and genotoxic potential of AA, PEN A and 3-ADON individually and in combination. Based on the MN frequency results in SH-SY5Y cells, AA demonstrated the most pronounced genotoxic effect among the individual treatments; while binary combinations, [PEN A + 3-ADON] had the highest MN frequency increase. Overall, the tertiary combination [AA + PEN A + 3-ADON] at the highest concentration exhibited the strongest genotoxic effect. This study highlights the significant genotoxic effects of mycotoxins, both individually and in combination, particularly in terms of micronucleus induction. Regarding mutagenicity, no activity was observed in the Ames test, however the increase in MN frequency indicates that these compounds can cause chromosomal damage, especially when combined. The findings underscore the importance of further research into mycotoxin combinations, as their combined effects may pose significant health risks. Simulating more realistic exposure scenarios, and complex mixtures, will be crucial for comprehensive risk assessment and ensuring food safety.

5.GENERAL DISCUSSION

5. Discusión general

DISCUSSION

To achieve the objectives of this Doctoral Thesis, a literature review was first conducted on the toxicological effects that AA and mycotoxins may induce on human health. Subsequently, using assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isobologram analysis and flow cytometry, the harmful effects of AA and mycotoxins (PEN A and FTC) on *in vitro* neuronal cell lines were evaluated, as well as the potential oxidative stress induction in SH-SY5Y cells by the ROS, LPO and GSH/GSSG ratio assays of AA, PEN A, and 3-ADON. Furthermore, the genotoxic potential of AA, PEN A, and 3-ADON, both individually and in combination, was investigated using micronucleus (MN) induction and the Ames test.

Among the AA, FTC, PEN A, and 3-ADON compounds, the effect of AA in SH-SY5Y has been widely studied (Forsby, 2011; Forsby et al., 1995; Hartley et al., 1997; Okuno et al., 2006; Sumizawa and Igisu, 2007, 2008, 2009), while no reports have been found on the study of FTC and PEN A in an in vitro culture of neuronal cells. In the present work, for the first time it has been reported the individually study of FTC and PEN A in SH-SY5Y cells as well as the AA, FTC, and PEN A combined effect in human neuroblastoma SH-SY5Y cells in relation to time mixture and compound exposure. AA has been classified as group 2A by the IARC. AA and its metabolite glycidamide form covalent adducts with DNA in mice and rats. In addition, AA causes genetic mutations and chromosomal aberrations in rodent somatic cells in vivo, cultured cells in vitro, and mouse germ cells.

Regarding cytotoxicity, several authors studied AA, in SH-SY5Y cells reporting effects for doses (0–10 mM) and exposure time (from 4 to 72h) similar to ours (Song et al., 2017; Yan et al., 2019, 2022). FTC showed no cytotoxic effect as shown in other studies in which they assayed mitoxantrone-selected human colon carcinoma cell line (S1-M1-3.2), for 72 h, individually and in combination to other compounds, it was shown that it could increase the drug accumulation in resistant cells, likely blocking the action of a putative drug transporter protein and allowed the cytotoxic agent to reach lethal levels (Sridhar K. Rabindran et al., 1998) According to the IC_{50} values of single compounds,

PEN A was the most cytotoxic mycotoxin compared to the other compounds assayed individually, similarly to the values obtained by (Bünger et al., 2004) who studied the cytotoxicity of PEN A individually, among other mycotoxins, in 4 different types of cells (L-929 A-549 Neuro-2a and Hep-G2), with IC_{50} values of 34.2, 20.5, 10.7 and 21.1 μM, respectively. Compared to our results their lower IC_{50} values are likely attributable to the different cell types used and the lower concentration ranges tested. In the case of binary combinations, the co-exposure of two compounds generally attenuated cytotoxicity in SH-SY5Y cells, as evidenced by increased IC_{50} values. In fact, most combinations led to higher cell viability than the corresponding individual treatments, except for the AA + PEN A combination and the triple mixture, both of which showed stronger cytotoxic responses. To date, no studies have been identified evaluating the combined effects of AA, FTC, and PEN A; however, the importance of assessing AA in combination with other mycotoxins, such as ochratoxin A (OTA), has been emphasized in various cell models including human kidney (HK-2), liver (HepG2), and intestinal (Caco-2) cells (Pyo et al., 2020; Su et al., 2023).

Given this context, the potential mechanisms underlying the neurotoxic effects induced by AA, FTC, and PEN A warrant further investigation. Specifically, for the AA + PEN A combination, previous research by Bridgeman et al. (2023) demonstrated that each compound, when tested individually, elicited a stronger cytotoxic response than their combination—an effect also reported by other authors (Agahi et al., 2020c). In contrast, no studies have been found regarding the effects of 3-ADON on SH-SY5Y cells, either individually or in combination with AA and PEN A.

On the other hand, several studies have stated the importance of studying the interactions between AA combined to other mycotoxin as well as the combination among mycotoxins (Bridgeman et al., 2023; Mitchell et al., 2024; Pyo et al., 2020; Su et al., 2023). When compared the 3-ADON with the double and tertiary combination of AA and PEN A, it can be stated that the tertiary combination was the most cytotoxic combination, followed by the double combination of [AA + 3-ADON], and then [PEN A + 3-ADON], the less cytotoxic was 3-ADON individually.

The cell cycle is a tightly regulated series of events through which cellular components are duplicated and equally distributed into daughter cells. In eukaryotic cells, DNA replication is restricted to the synthesis phase (S-phase), while chromosome segregation occurs during mitosis (M-phase). In this study, a general decrease in the G_0/G_1 phase was observed across all treatments, suggesting the induction of cell death at the concentrations tested—consistent with the cytotoxicity results obtained. Specifically, for AA, a marked reduction in the G_0/G_1 population was noted, in agreement with previous findings. Sumizawa and Igisu (2007), for instance, reported an increased proportion of cells in the sub-G_1 phase following AA exposure in SH-SY5Y cells, as determined by flow cytometric analysis, indicating DNA fragmentation typically associated with apoptosis.

Furthermore, in the same study they analysed the caspase-3 activation and how its suppression by Z-VAD-fmk diminished the cytotoxicity. These results obtained by Sumizawa and Igisu. (2007) indicate that apoptosis may be involved in the genesis of toxicity of AA in SH-SY5Y cells. Thus, to our knowledge, our results in the cell cycle disruption might be to the fact that AA induces apoptosis as Sumizawa and Igisu. (2007) stated that AA can activate caspase-3 and cause apoptosis in neuronal cells. In accordance with the results from cell cycle analysis presented for SH-SY5Y cells, after 24 h of exposure to FTC, cell proliferation was arrested remarkably in G0/G1 phase by the mycotoxin in comparison with non-treated cells. More studies are needed in other to fully understand the mechanisms of action of FTC. Regarding the cell cycle alteration induced by PEN A, we can also observe a diminution in the cell distribution in G0/G1, this can also be caused by the capacity of PEN A to induce apoptosis. Some studies, have state that PEN A can upregulate the TNF-α and given that TNF-α is a major cytokine known to induce cancer cell death through sustained JNK-activation, it can be concluded that the antiproliferative activity of PEN A is associated, in part, with the upregulation of TNF-α and subsequent activation of apoptotic cell death, as stated in other studies (Balkwill, 2009; Goda et al., 2018; Lin and Wang, 2008). This study provides critical insights into the cytotoxic effects of AA, FTC, PEN A and 3-ADON on SH-SY5Y cells. The findings emphasize the importance of studying mycotoxins

individually and in combination, as well as understanding their impact on cell cycle progression due to the fact that humans are exposed to a large number of mycotoxins simultaneously. These insights have implications for food safety standards and highlight the potential risks associated with exposure to these food contaminants in a more realistic scenario of mycotoxins with other food contaminants. However, more research in this area is essential for a comprehensive understanding of their effects on human health and further studies must be conducted to determine the cytotoxic mechanism of action of PEN A and FTC, as well as the interaction between AA, FTC and PEN A.

The negative impact of AA and PEN A in the nervous system of humans and animals has been previously reported (2014; Rundberget et al., 2010; Zhao, 2022). While for 3-ADON it has been reported negative impacts in different cell lines (Alassane-Kpembi et al., 2015; Juan-García et al., 2019a, 2016; Pinton et al., 2012) but never in a neuronal cells/system neither the combination of AA, PEN A, and 3-ADON. The present work reports for the first time the individual study of 3-ADON in SH-SY5Y cells as well as the AA, PEN A, and 3-ADON combined effect in human neuroblastoma SH-SY5Y cells in relation to time, mixture, and compound exposure. Therefore, the aim of this work was to stablish the impact of food natural and processed contaminants individually and in combination in the oxidative stress response in human neuroblastoma, SH-SY5Y cells. In general terms, the present study reveals an increase in the LPO and a decrease in the GSH/GSSG ratio for the combinations where AA intervene and respect to AA individual exposure, confirming the oxidative stress response. Therefore, these findings suggest that the combined effect induced higher lipid peroxidation in SH-SY5Y cells and with higher toxic effect than the individual treatment of AA. Nevertheless, for this compound (AA) the effect in ROS production diminished, which could be attributed to the differing exposure times used in the two assays, and that the cocktail of mycotoxins had a more complex behaviour, than the individual exposure. ROS production was assessed within 2 hours at sublethal concentrations, whereas LPO levels and GSH/GSSG ratio were measured after 24 hours of treatment.

Regarding the ROS results obtained for AA individually several authors have stablished that AA induces oxidative stress by increasing the ROS production and decreasing the GSH (Pan et al., 2018; Zhao et al., 2017) in line with our results. Nevertheless, other authors (Johansson et al., 2024) have stablished that after 9 days of exposure of AA at non-cytotoxic concentrations (0.33 μM and 10 μM) did neither decrease the level of GSH or total glutathione levels (GSH + GSSH), nor increased ROS production for the same cell line as that reported in this study. ROS results for PEN A, were similar to those obtained in other neuronal cell lines (cerebellar granule neuron and human neutrophils), revealing increases at similar concentrations as those tested by us (10 μM, 25 μM and 50 μM) (Berntsen et al., 2013). While for 3-ADON, it has been reported no increase of ROS in HepG2 cells at a concentrations bellow those tested here, which it does coincides with the results reported in here (Juan-García et al., 2019a).

ROS generation in binary combinations [AA + PEN A], [PEN A + 3-ADON] and their tertiary, if compared to individual treatments, there was always a decrease of the ROS production. Therefore, it seems like the individual treatments induced more ROS than the combination, a similar tendency was obtained by other authors when exposing SH-SY5Y and other cell lines to mixtures of mycotoxins; as it is the case of Fumonisin B1 (FB1) and Ochratoxin A (OTA) in SH-SY5Y (Penalva-Olcina et al., 2023), beauvericin (BEA) and OTA in HepG2 (Juan-García et al., 2020), or for zearalenone (ZEA) and aflatoxin B1 (AFB1) in cell porcine kidney cells suggesting an antagonistic effect in oxidative damage (Lei et al., 2013). No studies have been found for the triple combination of AA + PEN A + 3-ADON in the oxidative stress response. Nevertheless, other studies, in the same cell line, carried out tertiary combination of different mycotoxins, such as α-ZEL + β-ZEL + BEA, where a decrease in ROS generation was observed (Agahi, et al., 2020), similar to our results, where individual exposure induced more ROS generation than the tertiary combination.

Regarding the effect on the LPO an increase of MDA in SH-SY5Y cells exposed to AA was consistent with the result in bibliography in which it was state an increase in the LPO marker levels in different tissues and cell lines upon AA exposure (Allam et al.,

2013; Yousef and El-Demerdash, 2006). Furthermore, it has been suggested that the enhancement of LPO is a consequence of glutathione depletion to certain critical levels (Srivastava et al., 1983). The increase of MDA in PEN A and 3-ADON is aligned with the previous results obtained for other mycotoxins in different cell lines (Mézes et al., 2021). On the contrary, there was no increase in the MDA production after 24 h in HepG2 cells (Juan-García et al., 2019a), this difference may be due to the different concentrations tested or the different cell line. In binary combinations [AA + PEN A], [AA + 3-ADON] and [PEN A + 3-ADON], it was observed in general terms an increase of MDA if compared to each compound tested individually in line with other studies that stated that the mixture of mycotoxins increased the lipid peroxidation in Laying Hens (Kulcsár et al., 2024).

For the tertiary exposure [AA + PEN A + 3-ADON] levels of MDA compared to the individual treatment where different according to the compounds as it increased at all concentrations if compared to AA, opposite to what is was observed for PEN A as a decrease was observed; while for 3-ADON an oscillation was observed according to the concentration tested (increase and decrease). It can be set that the combination showed an additive or synergetic tendency, similarly to a study carried out in Caco-2 cells for other mycotoxins where the binary mixtures of deoxynivalenol (DON) with ZEA or fumonisin B1 (FB1) increased MDA production in a synergistic way whereas mixture of [ZEA + FB1] and mixture of all three mycotoxins show additive effects after 24h of exposure (Kouadio et al., 2007). Similarly, BEA and OTA increased the MDA production individually and in combination in HepG2 cells(Juan-García et al., 2020). Also, in HepG2, the combinations of DON + 3-ADON and 15-ADON revealed an increase in MDA production in mixtures or in combination of all three mycotoxins (Juan-García et al., 2019).

It is known that AA is oxidized to glycidamide, a reactive epoxide, and undergoes conjugation with reduced glutathione (GSH) (Ghanayem et al., 2005). DNA adducts from glycidamide have been reported following AA administration (Doerge et al., 2005; Dybing and Sanner, 2003). Combined exposures to AA, PEN A, and 3-ADON caused

a more pronounced reduction in the GSH/GSSG ratio across various concentrations compared to individual treatments. The tertiary mixture showed the most significant reduction, especially at high and low concentrations. These findings suggest that the combined exposure decrease of intracellular GSH at the highest concentrations and a significant increase of GSSG levels at lowest concentrations in SH-SY5Y cells as a response to oxidative damage. Similar tendency was obtained for different mycotoxins combinations in porcine kidney cells (Klarić et al., 2007). On the contrary, in the same cell line, but different mycotoxins (Beauvericin (BEA), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), there was an increase in the GSH/GSSG ratio for all the treatments individually and in combination (Agahi, et al., 2020). While for the same mycotoxins (α-ZEL + β-ZEL + BEA) the GPx activity obtained a higher increase in individual exposure than in combinations (Agahi, et al., 2020).

The results have significant implications for understanding how mycotoxin interactions contribute to oxidative stress and cellular damage in neuronal systems, as well as drawing attention to the need for regulatory considerations regarding combined mycotoxin exposure in food.

One step further, in this study, to assess the genotoxic ability of potential food contaminants, we used two short-term *in vitro* assays directed at different genotoxic endpoints in different phylogenetic systems: i) the MN assay, a commonly used technique to evaluate DNA damage at a chromosome level; and ii) the Ames test, a well-accepted tool for the assessment of the mutagenicity of chemicals. The combination of both tests fulfils the basic requirements to cover the three genetic endpoints to stablish if a substance is related to genotoxic or mutagenic effects; the *in vitro* MN test covers structural chromosome aberrations while the Ames test (bacterial reverse mutation assay) covers gene mutations (Benford et al., 2022). These two tests are effective for identifying most potential genotoxic substances. Adding other *in vitro* tests to them would notably decrease specificity without providing a meaningful improvement in sensitivity (Benford et al., 2022).

Concerning the MN assay conducted according to the methods described in TG OECD 487 (OECD/OCDE 487, 2023) and previously used in mycotoxins studies by Juan García *et al.,* (Juan-García et al., 2019b, 2018), we observed an increase in the MN frequency at all the concentrations assayed for AA, PEN A and 3-ADON, as well as for the combinations. Regarding AA, similar results were obtained in human lymphocytes by Zamani et al., (2018) with a significant increase in frequency of MN at concentrations of 50 and 100 mM. In another study, conducted in mouse bone marrow cells, AA significantly increased the frequency of micronucleated cells in exposed groups compared to the negative control, supporting again that AA may induce genotoxicity (Algarni, 2018). Further investigation into AA has revealed species-specific differences *in vivo*. For instance, a dose-dependent increase in MN formation was observed in mice but not in rats, potentially due to higher glycidamide levels in mice (Hobbs et al., 2016). Additionally, AA caused 10- and 20-fold increases in MN frequencies in morphologically normal and abnormal mouse embryos, respectively, with 41 and 93 MN per 1,000 cells (Titenko-Holland et al., 1998). These findings are in line with the trend observed in our results, albeit in SH-SY5Y cells.

Regarding genotoxicity induced by mycotoxin exposure, a wide range of toxins and cell lines have been studied. According, MN formation was assessed in HepG2 cells, bone marrow polychromatophilic erythrocytes, and neuroblastoma SH-SY5Y cells, for the following mycotoxins: 15-acetyldeoxinyvalenol (15-ADON), aflatoxin B1 (AFB1), beauvericin (BEA), deoxynivalenol (DON), ochratoxin A (OTA), T-2 toxin, sterigmatocystin (STE) and patulin (PAT) ((Juan-García et al., 2019, 2018; Šmerák et al., 2001; Taroncher et al., 2024; Zingales et al., 2021) as well as in bone marrow cells, rabbit kidney cells, and human lymphocytes for fumonisin B1 (FB1) and citrinin (CTN) (Dönmez-Altuntas et al., 2007; Rumora et al., 2002; Song et al., 2014; Zhou et al., 2022). In detail, Juan García et al. (2018) stated that at 3 µM of 3-ADON, an increase of 13.1 % of MN was obtained compared to control; furthermore, at a range of 0.6 to 4,8 µM for DON, and of 0.2 to 1.5 µM for both, 3-ADON and 15-ADON the binary combinations of [DON + 3-ADON] and [DON + 15-ADON] also increased MN frequency in the range from 12.0 % to 23.8 % even at the lowest concentrations tested;

while for [3-ADON + 15-ADON] the increase was more noticeable; reaching values of 43.8 % (Juan-García et al., 2018). On the other hand, for tertiary combinations [DON + 3-ADON + 15-ADON], all concentrations assayed revealed increases in MN frequencies from 31.2 % to 46.7 % with respect to the control (Juan-García et al., 2018), being closer to those reported in this study for SH-SY5Y cells. Also, similar outcomes (an increase of 12.1 %) was obtained for T-2 toxin in HepG2 cells, at the highest concentration assayed (60 nM) (Taroncher et al., 2024). Coinciding with our cell line, there is only one study in which genotoxicity and MN induction have been investigated with STE mycotoxin, obtaining an increase 4 % with respect to the control (Zingales et al., 2021). This tendency is consistent with those reported in our work, in which a clear increase in MN formation was observed in all individual treatments. Moreover, PAT significantly increased MN frequency by 35% in human hepatoma HepG2 cells at the highest tested concentration (0.75 μM) (Zhou et al., 2009) and induced chromosomal aberrations in bone marrow cells (Song et al., 2014). Similarly, FB1 exposure led to a concentration- and time-dependent increase in MN frequency in rabbit kidney RK13 cells, ranging from 24 to 49 per 1,000 cells (Rumora et al., 2002). Additionally, CTN induced a significant, concentration-dependent increase in MN frequency, ranging from 2.07% to 4.73% at a concentration of 10 to 100 μM, in human lymphocytes (Dönmez-Altuntas et al., 2007). While studies of BEA and OTA have revealed increases of 14.2 % and 12 % for BEA (1.25 μM) and OTA (25 μM), respectively in HepG2 cells; whereas combined ([BEA + OTA]) the increase in the MN frequency ranged from 24 % to 28.3 % compared to the control (Juan-García et al., 2019), all this cases similarly to our results. Among mycotoxins, AFB1 is undoubtedly the most genotoxic and although far from our results, indeed, AFB1 can promote in HepG2 an increase of 80% in MN formation compared to the control (at 9.6 μM AFB_1) (Singto et al., 2020)

Moving to combined exposure, the MN study of AFB1 jointly OTA has been carried out in bone marrow cells (Corcuera et al., 2015). For AFB1, MN induction was significantly high (up to 90 times the control levels), while no effect was reported for OTA (Corcuera et al., 2015). In that same study for combined treatment ([AFB1 + OTA]) a noticeable MN induction was reached (69 times the control levels) although it

was lower than when AFB1 was individually tested, indicating a possible antagonist effect (Corcuera et al., 2015). Similar results were observed in our study, wherein the addition of AA to mycotoxins reduced MN frequency, suggesting a possible interference of AA in mycotoxins induced genotoxicity. One step further, in an animal model, it was found a significantly higher frequency of MN upon exposure to [T-2 toxin + AFB1] in comparison with the effect of AB1 alone (Šmerák et al., 2001). These outcomes align with our findings in which [PEN A + 3-ADON] showed an additive effect on MN promotion. The reason of the effects observed in combinations treatment in our study can be associated to some implications of AA, 3-ADON or PEN-A in chromosome lagging that makes to have an inconsistency profile according to the doses and exposure time tested. Nevertheless, further investigations are needed to confirm such mechanism trigger by these contaminants.

Altogether, AA, PEN A, and 3-ADON, in both individual and combined treatments, demonstrated MN induction levels comparable to or exceeding those reported for other mycotoxins in various models, highlighting their potent genotoxic potential. These findings emphasize the importance of evaluating food contaminants both individually and in combination to better understand their cumulative effects.

Although the mutagenicity of AA, PEN A, and 3-ADON has been previously studied (Bull et al., 1984; Eisenbrand, 2020b; Hashimoto and Tanii, 1985; Sabater-Vilar et al., 2003; Wehner et al., 1978) no studies have evaluated their combined effects. Our negative mutagenic results align with earlier findings, which consistently demonstrated no mutagenic potential for these substances individually, with or without S9 activation, across various strains. In particular, for AA previous studies reported no mutagenicity in *Salmonella* strains TA1535, TA1537, TA1538, TA98, and TA100, both in the presence and absence of metabolic activation (Bull et al., 1984; Hashimoto and Tanii, 1985). However, Crudo et al. (2023) observed a slight mutagenic effect for AA at 50 mM (10 times higher than concentrations used in our study) in TA100 in the presence of S9 activation, we obtained a similar tendency in TA1537 with metabolic activation, probably because is metabolized by cytochrome P450 2E1 to its reactive metabolite

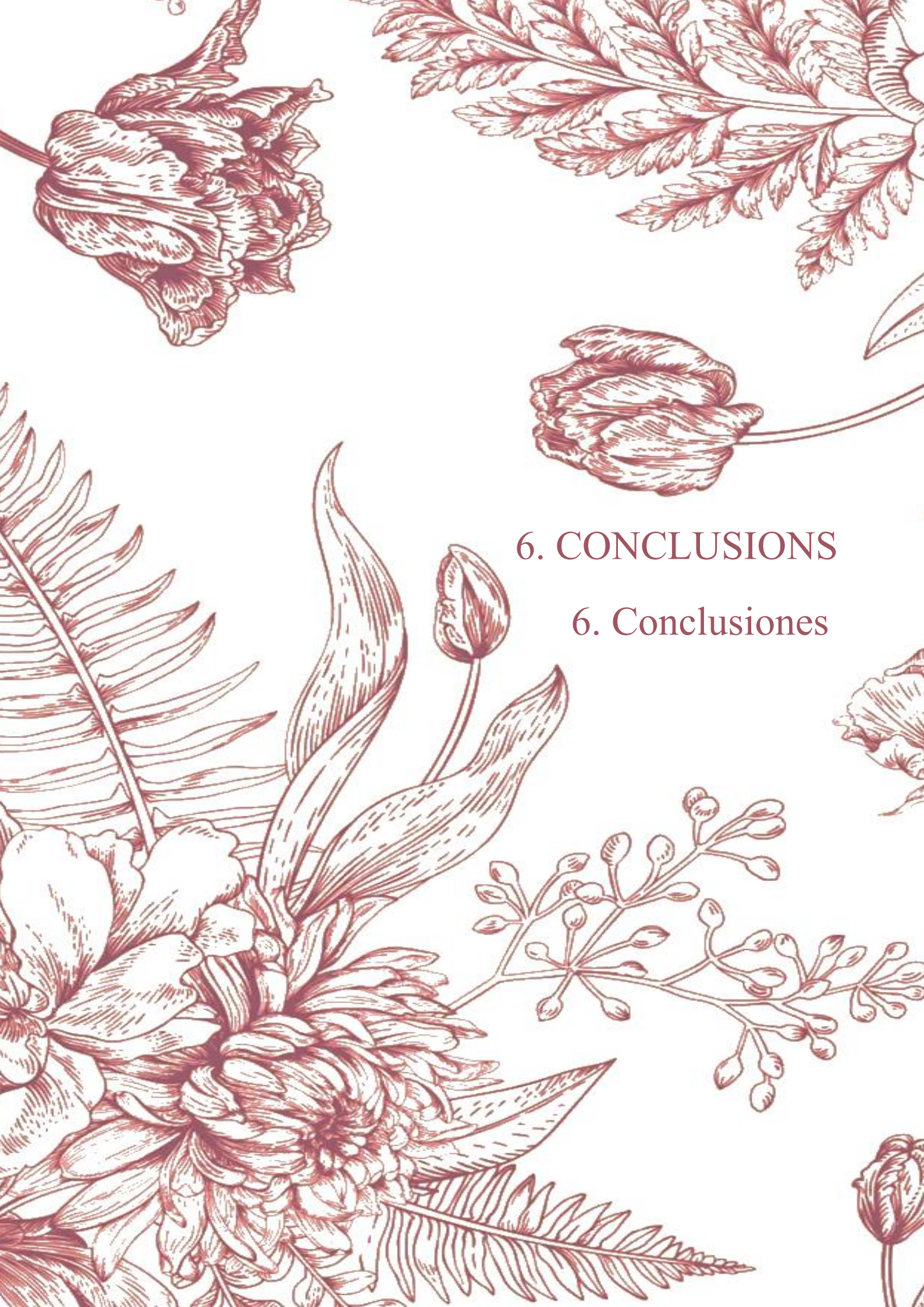
glycidamide (Hölzl-Armstrong et al., 2020). Although differences in strains and methodologies exist (such as the use of *E. coli* instead of *Salmonella* in this study) our findings are consistent with the literature. Regarding PEN A, using the Ames test with *Salmonella* strains (TA1535, TA1537, TA1538, TA98, and TA100) with and without metabolic activation, Sabater-Vilar et al. (2003) reported no mutagenic effects, consistent with our findings using *E. coli* WP2. Similarly, mutagenicity assays for 3-ADON at 5 times higher concentrations in the same *Salmonella* strains than ours, showed no mutagenic activity, aligning with our work, despite differences in the bacterial systems used (Wehner et al., 1978)

Regarding the combination scenarios tested, it was not obtained mutagenicity at any combination or concentration tested. These findings could be related to the concentration tested, which mimics a wide range of real scenarios of exposure but are insufficient to see any mutagenic impacts. Similarly to our results, Ehrlich et al., (2002) obtained a MN positive induction in a dose-dependent manner for FB1, and an absence of revertant colonies in the Ames test under all test conditions for the TA98, TA100, TA102, TA1535, 1537 stains. Regarding combinations of AA and mycotoxins, similar results were obtained in the study of mutagenicity and genotoxicity from AA with the *Alternaria* mycotoxin alternariol (AOH). The combination did not show synergistic effects during the combined treatments in TA98 and TA100 at similar concentrations of AA and 0.1 and 10 µM of AOH. However, it did show slight mutagenic effects, both in the presence and absence of the S9 fraction, at a concentration 10 times higher than ours (50 µM + 0.5/50 mM) of [AOH + AA] (Crudo et al., 2023).

Concerning the mycotoxins combinations, to our knowledge just trichothecenes and AFB1 mixtures have been carried out. For T-2 toxin and vomitoxin it was not showing mutagenic activity at the concentrations assayed 60 nM and 0.43 µM for T2 and 5.4µM for vomitoxin (Šmerák et al., 2001; Taroncher et al., 2024), similar to our work. However, in combination with AFB_1, authors reported a mutagenic effect significantly greater than AFB_1 alone in the Ames test (in strain TA98 at all concentrations) (Šmerák et al., 2001). In this case, the most mutagenic mixture was [AFB1 + T-2 + vomitoxin]

(Šmerák et al., 2001). The dissonance of results is probably due to the fact that they used different mycotoxins than ours, specially AFB_1 that is genotoxic and mutagenic with metabolic activation, as previously stated in literature (Aydin and Rencüzoğullari, 2019). While the mutagenic effects of AFB_1 have been extensively studied (Šmerák et al., 2001) and linked to its metabolite 8,9-epoxide-aflatoxin B1, inducing chromosomal aberrations, micronuclei formation, and other genotoxic outcomes, the mutagenic potential of the mycotoxins studied in this work remains unknown.

The absence of mutagenic response in the Ames test observed for AA, PEN A, 3-ADON, and their combinations aligns with our current findings. However, to our knowledge, no data are available in the literature regarding the mutagenic potential of these specific mycotoxin combinations or their interaction with AA, particularly in the bacterial strains used in this study. Therefore, more studies are needed in order to better understand the mechanism of action underlying the genotoxicity of these compounds. Highlighting the need for further research, as combined mycotoxins with food processed contaminants could be a genuine health risk to the human population.



6. CONCLUSIONS

6. Conclusiones

6. CONCLUSIONES

1. La literatura revisada muestra que la AA y diversas micotoxinas comparten efectos neurotóxicos comunes, especialmente a nivel celular en células SH-SY5Y, actuando de manera dependiente del tiempo y la dosis. Los ensayos más utilizados para estudiar su toxicidad incluyen MTT, apoptosis y marcadores de estrés oxidativo. Ambos tipos de compuestos modulan vías clave relacionadas con la inflamación, la apoptosis y la diferenciación neuronal. Varios compuestos bioactivos naturales, como la curcumina, la cafeína y extractos vegetales, han mostrado efectos protectores frente a su toxicidad.

2. AA y PEN A exhibieron efectos citotóxicos, siendo PEN A el compuesto con mayor potencia citotóxica entre los analizados (AA y FTC). La interacción principal observada entre los compuestos en todas las combinaciones analizadas fue de tipo aditivo.

3. AA y PEN A alteraron el ciclo celular de las células SH-SY5Y. Entre todos los compuestos evaluados, PEN A mostró el efecto más fuerte sobre la interrupción del ciclo celular, provocando una detención en la fase G0/G1. Esta detención sugiere el potencial de PEN A para inducir apoptosis en las células SH-SY5Y.

4. El estrés oxidativo parece desempeñar un papel clave en la citotoxicidad inducida por AA, PEN A y 3-ADON, tanto de forma individual como combinada. Las exposiciones combinadas provocaron un aumento de la peroxidación lipídica y una reducción en la relación GSH/GSSG, lo que sugiere posibles efectos sinérgicos o aditivos. Las mezclas de estos contaminantes parecen ser más tóxicas que las exposiciones individuales, lo que resalta la necesidad de seguir estudiando estas combinaciones.

5. AA mostró el efecto genotóxico más pronunciado entre los tratamientos individuales, según los resultados de frecuencia de micronúcleos (MN) en

células SH-SY5Y. La combinación binaria de [PEN A + 3-ADON] produjo el mayor aumento en la frecuencia de MN. El efecto genotóxico más alto se observó con la combinación terciaria de [AA + PEN A + 3-ADON] a la concentración más elevada. El estudio subraya los importantes efectos genotóxicos de estas micotoxinas, especialmente en cuanto a la inducción de micronúcleos. No se observó actividad mutagénica en el test de Ames.

6. Este trabajo ha contribuido significativamente a consolidar y comparar la evidencia científica más reciente sobre los efectos citotóxicos, genotóxicos y mutagénicos del AA, PEN A y 3-ADON, tanto individualmente como en combinación, en modelos celulares neuronales humanos. Mediante la recopilación y el análisis sistemáticos de los mecanismos celulares implicados, como la interrupción del ciclo celular, el estrés oxidativo, la formación de micronúcleos y la mutagenicidad, se proporciona una base sólida para una mejor comprensión de los riesgos potenciales asociados a estas sustancias presentes en los alimentos. Además, destaca las interacciones aditivas o sinérgicas entre contaminantes, un área aún poco explorada y de gran relevancia para la evaluación de riesgos alimentarios.

7. Se debe seguir investigando para explorar otros mecanismos intracelulares implicados en la toxicidad inducida por AA, PEN A y 3-ADON. Los hallazgos destacan la necesidad de seguir investigando combinaciones de micotoxinas, ya que sus efectos combinados podrían representar riesgos importantes para la salud. Simular escenarios de exposición realistas y mezclas complejas es esencial para una evaluación precisa del riesgo y para garantizar la seguridad alimentaria.

6. CONCLUSIONS

1.- The reviwed literature shows that AA and various mycotoxins share common neurotoxic effects, especially at the cellular level in SH-SY5Y cells, acting in a time- and dose-dependent manner. The most frequently used assays to study their toxicity include MTT, apoptosis, and oxidative stress markers. Both types of compounds modulate key pathways related to inflammation, apoptosis, and neuronal differentiation. Several natural bioactive compounds, such as curcumin, caffeine, and plant extracts, have shown protective effects against their toxicity.

2.- AA and PEN A exhibited cytotoxic effects, with 3-ADON showing the highest cytotoxic potency among the tested compounds (AA, PEN A and FTC). The primary interaction detected between compounds in all tested combinations was additive.

3.- AA and PEN A disrupted the cell cycle of SH-SY5Y cells. PEN A had the strongest effect on cell cycle disruption, causing cell cycle arrest in the G0/G1 phase. The G0/G1 arrest induced by PEN A suggests its potential to trigger apoptosis in SH-SY5Y cells.

4.- Oxidative stress appears to play a key role in the cytotoxicity induced by AA, PEN A, and 3-ADON, both individually and in combination. Combined exposures induced higher lipid peroxidation and reduced GSH/GSSG ratios, suggesting potential synergistic or additive effects. Mixtures of these contaminants seem to be more toxic than individual exposures, highlighting the need to study such combinations further.

5.- AA showed the most pronounced genotoxic effect among the individual treatments, based on the MN frequency results in SH-SY5Y cells. The binary combination of [PEN A + 3-ADON] produced the highest increase in MN frequency. The highest genotoxic effect was observed in the tertiary combination of [AA + PEN A + 3-ADON] at the highest concentration. This emphasizes the significant genotoxic effects of these mycotoxins, particularly in terms of micronucleus induction. No mutagenic activity was observed in the Ames test.

6.- This work has significantly contributed to consolidating and comparing the most recent scientific evidence on the cytotoxic, genotoxic, and mutagenic effects of AA, PEN A, and 3-ADON, both individually and in combination, in human neuronal cell models. By systematically compiling and analyzing the cellular mechanisms involved, such as cell cycle disruption, oxidative stress, micronucleus formation and mutagenicity, a solid foundation is provided for a better understanding of the potential risks associated with these substances present in foods. Furthermore, it highlights additive or synergistic interactions between contaminants, a still underexplored area of great relevance for food risk assessment.

7.- Additional research is necessary to explore other intracellular mechanisms involved in AA, PEN A, FTC and 3-ADON-induced toxicity. The findings highlight the need for further research on mycotoxin combinations, as their combined effects may present significant health risks. Simulating realistic exposure scenarios and complex mixtures is essential for accurate risk assessment and ensuring food safety.

7. BIBLIOGRAPHY

7. Bibliografía

7. Bibliography

Acrylamide (IARC Summary & Evaluation, Volume 60, 1994) Available online: https://inchem.org/documents/iarc/vol60/m60-11.html (accessed on 29 June 2023).

Agahi, F., Álvarez-Ortega, N., Font, G., Juan-García, A., Juan, C., 2020a. Oxidative stress, glutathione, and gene expression as key indicators in SH-SY5Y cells exposed to zearalenone metabolites and beauvericin. Toxicol Lett 334, 44–52. https://doi.org/10.1016/J.TOXLET.2020.09.011

Agahi, F., Font, G., Juan, C., Juan-García, A., 2020b. Individual and Combined Effect of Zearalenone Derivates and Beauvericin Mycotoxins on SH-SY5Y Cells. Toxins 2020, Vol. 12, Page 212 12, 212. https://doi.org/10.3390/TOXINS12040212

Alassane-Kpembi, I., Puel, O., Oswald, I.P., 2015. Toxicological interactions between the mycotoxins deoxynivalenol, nivalenol and their acetylated derivatives in intestinal epithelial cells. Arch Toxicol 89, 1337–1346. https://doi.org/10.1007/S00204-014-1309-4/TABLES/2

Algarni, A.A., 2018. Genotoxic effects of acrylamide in mouse bone marrow cells. Caryologia 71, 160–165. https://doi.org/10.1080/00087114.2018.1450801

Allam, A., El-Gareeb, A., Ajarem, J., Abdul-Hamid, M., El-bakry, A., 2013. Effect of acrylamide on the development of medulla oblongata in albino rat: Biochemical and morphological studies. Afr J Pharm Pharmacol 7, 1320–1331. https://doi.org/10.5897/AJPP12.1193

Allam, A., El-Ghareeb, A., Abdul-Hamid, M., Baikry, A., Sabri, M., 2011. Prenatal and perinatal acrylamide disrupts the development of cerebellum in rat: Biochemical and morphological studies. http://dx.doi.org/10.1177/0748233710386412 27, 291–306. https://doi.org/10.1177/0748233710386412

AMPCo Tremor Syndrome Associated with a Fungal Toxin: Sequelae of Food Contamination; Tremor Syndrome Associated with a Fungal Toxin: Sequelae of Food Contamination., doi:10.5694/j.1326-5377.2005.tb06819.x.

Aydin, M., Rencüzoğullari, E., 2019. Genotoxic and Mutagenic Effects of Mycotoxins: A Review. Comm. J. Biol 3, 132–161. https://doi.org/10.31594/commagene.633418

Ayoubi, C., Alonso-Jauregui, M., Azqueta, A., Vignard, J., Mirey, G., Rocher, O., Puel, O., Oswald, I.P., Vettorazzi, A., Soler, L., 2023. Mutagenicity and genotoxicity assessment of the emerging mycotoxin Versicolorin A, an Aflatoxin B1 precursor. Environmental Pollution 335, 122276. https://doi.org/10.1016/J.ENVPOL.2023.122276

Balkwill, F. Tumour Necrosis Factor and Cancer. Nature Reviews Cancer 2009 9:5 2009, 9, 361–371, doi:10.1038/nrc2628.

Benford, D., Bignami, M., Chipman, J.K., Ramos Bordajandi, L., 2022. Assessment of the genotoxicity of acrylamide. EFSA Journal 20, e07293. https://doi.org/10.2903/J.EFSA.2022.7293

Berntsen, H.F., Bogen, I.L., Wigestrand, M.B., Fonnum, F., Walaas, S.I., Moldes-Anaya, A., 2017. The fungal neurotoxin penitrem A induces the production of reactive oxygen species in human neutrophils at submicromolar concentrations. Toxicology 392, 64–70. https://doi.org/10.1016/J.TOX.2017.10.008

Berntsen, H.F., Wigestrand, M.B., Bogen, I.L., Fonnum, F., Walaas, S.I., Moldes-Anaya, A., 2013. Mechanisms of penitrem-induced cerebellar granule neuron death in vitro: Possible involvement of GABAA receptors and oxidative processes. Neurotoxicology 35, 129–136. https://doi.org/10.1016/J.NEURO.2013.01.004

Berntsen, H.F.; Bogen, I.L.; Wigestrand, M.B.; Fonnum, F.; Walaas, S.I.; Moldes-Anaya, A. The Fungal Neurotoxin Penitrem A Induces the Production of Reactive Oxygen Species in Human Neutrophils at Submicromolar Concentrations. Toxicology 2017, 392, 64–70, doi:10.1016/J.TOX.2017.10.008.

Breton, P.; Bizot, J.C.; Buee, J.; De La Manche, I. Brain Neurotoxicity of Penitrem A: Electrophysiological, Behavioral and Histopathological Study. Toxicon 1998, 36, 645–655, doi:10.1016/S0041-0101(97)00084-6.

Bridgeman, L., Juan, C., Berrada, H., Juan-García, A., 2024. Effect of Acrylamide and Mycotoxins in SH-SY5Y Cells: A Review. Toxins 2024, Vol. 16, Page 87 16, 87. https://doi.org/10.3390/TOXINS16020087

Bridgeman, L., Juan, C., Juan-García, A., Berrada, H., 2023. Individual and combined effect of acrylamide, fumitremorgin C and penitrem A on human neuroblastoma SH-SY5Y cells. Food and Chemical Toxicology 182, 114114. https://doi.org/10.1016/J.FCT.2023.114114

Brusick, D.J., Simmon, V.F., Rosenkranz, H.S., Ray, V.A., Stafford, R.S., 1980. An evaluation of the Escherichia coli WP2 and WP2uvrA reverse mutation assay. Mutation Research/Reviews in Genetic Toxicology 76, 169–190. https://doi.org/10.1016/0165-1110(80)90009-3

Bryce, S.M., Avlasevich, S.L., Bemis, J.C., Lukamowicz, M., Elhajouji, A., Van Goethem, F., De Boeck, M., Beerens, D., Aerts, H., Van Gompel, J., Collins, J.E., Ellis, P.C., White, A.T., Lynch, A.M., Dertinger, S.D., 2008. Interlaboratory evaluation of a flow cytometric, high content in vitro micronucleus assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 650, 181–195. https://doi.org/10.1016/J.MRGENTOX.2007.11.006

Bull, R.J., Robinson, M., Stober, J.A., 1984. Carcinogenic activity of acrylamide in the skin and lung of Swiss-ICR mice. Cancer Lett 24, 209–212. https://doi.org/10.1016/0304-3835(84)90138-1

Bünger, J.; Westphal, G.; Mönnich, A.; Hinnendahl, B.; Hallier, E.; Müller, M. Cytotoxicity of Occupationally and Environmentally Relevant Mycotoxins. Toxicology 2004, 202, 199–211, doi:10.1016/J.TOX.2004.05.007.

Burke, D.A., Wedd, D.J., Burlinson, B., 1996. Use of the Miniscreen assay to screen novel compounds for bacterial mutagenicity in the pharmacentical industry. Mutagenesis 11, 201–205. https://doi.org/10.1093/MUTAGE/11.2.201

Burton, G.J., Jauniaux, E., 2011. Oxidative stress. Best Pract Res Clin Obstet Gynaecol 25, 287–299. https://doi.org/10.1016/J.BPOBGYN.2010.10.016

Capuano, E., Fogliano, V., 2011. Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. LWT - Food Science and Technology 44, 793–810. https://doi.org/10.1016/J.LWT.2010.11.002

Carmen González, M., Lull, C., Moya, P., Ayala, I., Primo, J., Yúfera Primo, E., 2003. Insecticidal activity of penitrems, including penitrem G, a new member of the family isolated from Penicillium crustosum. J Agric Food Chem 51, 2156–2160. https://doi.org/10.1021/JF020983E

Chen, X., Xiao, J., Fu, H., Zhang, Y., Li, Y., Yang, H., Gao, W., Li, B., 2022. Acrylamide-induced damage to postsynaptic plasticity is CYP2E1 dependent in an SH-SY5Y co-culture system. Toxicology in Vitro 84, 105455. https://doi.org/10.1016/J.TIV.2022.105455

Chou, T.C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. Pharmacol Rev 2006, 58, 621–681, doi:10.1124/PR.58.3.10.

Chou, T.C.; Talalay, P. Quantitative Analysis of Dose-Effect Relationships: The Combined Effects of Multiple Drugs or Enzyme Inhibitors. Adv Enzyme Regul 1984, 22, 27–55, doi:10.1016/0065-2571(84)90007-4.

Cole, R.J.; Cox, R.H. Handbook of Toxic Fungal Metabolites; Academic Press, 1981; ISBN 9780121797607.

Corcuera, L.A., Vettorazzi, A., Arbillaga, L., Pérez, N., Gil, A.G., Azqueta, A., González-Peñas, E., García-Jalón, J.A., López de Cerain, A., 2015. Genotoxicity of Aflatoxin B1 and Ochratoxin A after simultaneous application of the in vivo micronucleus and comet assay. Food and Chemical Toxicology 76, 116–124. https://doi.org/10.1016/J.FCT.2014.12.003

Corvi, R., Albertini, S., Hartung, T., Hoffmann, S., Maurici, D., Pfuhler, S., Van Benthem, J., Vanparys, P., 2008. ECVAM retrospective validation of in vitro micronucleus test (MNT). Mutagenesis 23, 271. https://doi.org/10.1093/MUTAGE/GEN010

Crudo, F., Hong, C., Varga, E., Del Favero, G., Marko, D., 2023. Genotoxic and Mutagenic Effects of the Alternaria Mycotoxin Alternariol in Combination with the Process Contaminant Acrylamide. Toxins 2023, Vol. 15, Page 670 15, 670. https://doi.org/10.3390/TOXINS15120670

Deamer, D., 2017. The Role of Lipid Membranes in Life's Origin. Life 7. https://doi.org/10.3390/LIFE7010005

Doerge, D.R., Gamboa Da Costa, G., McDaniel, L.P., Churchwell, M.I., Twaddle, N.C., Beland, F.A., 2005. DNA adducts derived from administration of acrylamide and glycidamide to mice and rats. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 580, 131–141. https://doi.org/10.1016/J.MRGENTOX.2004.10.013

Dönmez-Altuntas, H., Dumlupinar, G., Imamoglu, N., Hamurcu, Z., Liman, B.C., 2007. Effects of the mycotoxin citrinin on micronucleus formation in a cytokinesis-block genotoxicity assay in cultured human lymphocytes. Journal of Applied Toxicology 27, 337–341. https://doi.org/10.1002/JAT.1209

Dorner, J.W.; Cole, R.J.; Hill, R.A. Food Chem. 1984, 32, 411–413.

Dybing, E., Sanner, T., 2003. Risk assessment of acrylamide in foods. Toxicol Sci 75, 7–15. https://doi.org/10.1093/TOXSCI/KFG165

EFSA, 2011. Scientific Opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. EFSA J. 9, 2407.

Ehrlich, V., Darroudi, F., Uhl, M., Steinkellner, H., Zsivkovits, M., Knasmueller, S., 2002. Fumonisin B1 is genotoxic in human derived hepatoma (HepG2) cells. Mutagenesis 17, 257–260. https://doi.org/10.1093/MUTAGE/17.3.257

Eisenbrand, G., 2020. Revisiting the evidence for genotoxicity of acrylamide (AA), key to risk assessment of dietary AA exposure. Arch Toxicol 94, 2939–2950. https://doi.org/10.1007/S00204-020-02794-3/FIGURES/1

Eriksen, G.S., Jäderlund, K.H., Moldes-Anaya, A., Schönheit, J., Bernhoft, A., Jæger, G., Rundberget, T., Skaar, I., 2010. Poisoning of dogs with tremorgenic Penicillium toxins. Med Mycol 48, 188–196. https://doi.org/10.3109/13693780903225821

Eriksen, G.S., Moldes-Anaya, A., Fæste, C.K., 2013. Penitrem A and analogues: Toxicokinetics, toxicodynamics including mechanism of action and clinical significance. World Mycotoxin J 6, 263–272. https://doi.org/10.3920/WMJ2013.1574

Erkekoglu, P.; Baydar, T. Acrylamide Neurotoxicity. Nutr Neurosci 2014, 17, 49–57, doi:10.1179/1476830513Y.0000000065.

Fernández-Bertólez, N., Brandão, F., Costa, C., Pásaro, E., Teixeira, J.P., Laffon, B., Valdiglesias, V., 2021. Suitability of the in vitro cytokinesis-block micronucleus test for genotoxicity assessment of tio2 nanoparticles on sh-sy5y cells. Int J Mol Sci 22, 8558. https://doi.org/10.3390/IJMS22168558/S1

Flamand, N., Meunier, J.R., Meunier, P.A., Agapakis-Caussé, C., 2001. Mini mutagenicity test: A miniaturized version of the Ames test used in a prescreening assay for point mutagenesis assessment. Toxicology in Vitro 15, 105–114. https://doi.org/10.1016/S0887-2333(01)00003-0

Forsby, A. Neurite Degeneration in Human Neuronal SH-SY5Y Cells as an Indicator of Axonopathy. Neuromethods 2011, 56, 255–268, doi:10.1007/978-1-61779-077-5_12/FIGURES/3_12.

Forsby, A.; Pilli, F.; Bianchi, V.; Walum, E. Determination of Critical Cellular Neurotoxic Concentrations in Human Neuroblastoma (SH-SY5Y) Cell Cultures. undefined 1995, 23, 800–811, doi:10.1177/026119299502300610.

Fu, Y.H., He, Y., Phan, K., Bhatia, S., Pickford, R., Wu, P., Dzamko, N., Halliday, G.M., Kim, W.S., 2022. Increased unsaturated lipids underlie lipid peroxidation in synucleinopathy brain. Acta Neuropathol Commun 10, 165. https://doi.org/10.1186/S40478-022-01469-7

García-Sánchez, A., Miranda-Díaz, A.G., Cardona-Muñoz, E.G., 2020. The Role of Oxidative Stress in Physiopathology and Pharmacological Treatment with Pro- and Antioxidant Properties in Chronic Diseases. Oxid Med Cell Longev 2020. https://doi.org/10.1155/2020/2082145

Garimella, T.S.; Ross, D.D.; Bauer, K.S. Liquid Chromatography Method for the Quantitation of the Breast Cancer Resistance Protein ABCG2 Inhibitor Fumitremorgin C and Its Chemical Analogues in Mouse Plasma and Tissues. J Chromatogr B Analyt Technol Biomed Life Sci 2004, 807, 203–208, doi:10.1016/J.JCHROMB.2004.04.008.

Ghanayem, B.I., McDaniel, L.P., Churchwell, M.I., Twaddle, N.C., Snyder, R., Fennell, T.R., Doerge, D.R., 2005. Role of CYP2E1 in the Epoxidation of Acrylamide to Glycidamide and Formation of DNA and Hemoglobin Adducts. Toxicological Sciences 88, 311–318. https://doi.org/10.1093/TOXSCI/KFI307

Goda, A.A.; Bakar Siddique, A.; Mohyeldin, M.; Ayoub, N.M.; El Sayed, K.A. Marine Drugs The Maxi-K (BK) Channel Antagonist Penitrem A as a Novel Breast Cancer-Targeted Therapeutic. 2018, doi:10.3390/md16050157.

Han, Z., Nie, D., Ediage, E.N., Yang, X., Wang, J., Chen, B., Li, S., On, S.L.W., De Saeger, S., Wu, A., 2014. Cumulative health risk assessment of co-occurring mycotoxins of deoxynivalenol and its acetyl derivatives in wheat and maize: Case study, Shanghai, China. Food and Chemical Toxicology 74, 334–342. https://doi.org/10.1016/J.FCT.2014.10.018

Hartley, C.L.; Anderson, V.E.R.; Anderson, B.H.; Robertson, J. Acrylamide and 2,5-Hexanedione Induce Collapse of Neurofilaments in SH-SY5Y Human Neuroblastoma Cells to Form Perikaryal Inclusion Bodies. Neuropathol Appl Neurobiol 1997, 23, 364–372, doi:10.1111/J.1365-2990.1997.TB01310.X.

Hartwig, A., Arand, M., Epe, B., Guth, S., Jahnke, G., Lampen, A., Martus, H.J., Monien, B., Rietjens, I.M.C.M., Schmitz-Spanke, S., Schriever-Schwemmer, G., Steinberg, P., Eisenbrand, G., 2020. Mode of action-based risk assessment of genotoxic carcinogens. Arch Toxicol 94, 1787. https://doi.org/10.1007/S00204-020-02733-2

Hashimoto, K., Tanii, H., 1985. Mutagenicity of acrylamide and its analogues in Salmonella typhimurium. Mutat Res 158, 129–133. https://doi.org/10.1016/0165-1218(85)90075-8

Hobbs, C.A., Davis, J., Shepard, K., Chepelev, N., Friedman, M., Marroni, D., Recio, L., 2016. Differential genotoxicity of acrylamide in the micronucleus and Pig-a gene mutation assays in F344 rats and B6C3F1 mice. Mutagenesis 31, 617–626. https://doi.org/10.1093/mutage/gew028

Hölzl-Armstrong, L., Kucab, J.E., Moody, S., Zwart, E.P., Loutkotová, L., Duffy, V., Luijten, M., Gamboa da Costa, G., Stratton, M.R., Phillips, D.H., Arlt, V.M., 2020. Mutagenicity of acrylamide and glycidamide in human TP53 knock-in (Hupki) mouse embryo fibroblasts. Arch Toxicol 94, 4173–4196. https://doi.org/10.1007/S00204-020-02878-0

Johansson, Y., Andreassen, M., Hartsch, M., Wagner, S., Forsby, A., 2024. Attenuated neuronal differentiation caused by acrylamide is not related to oxidative stress in differentiated human neuroblastoma SH-SY5Y cells. Food and Chemical Toxicology 187, 114623. https://doi.org/10.1016/J.FCT.2024.114623

Jomova, K., Raptova, R., Alomar, S.Y., Alwasel, S.H., Nepovimova, E., Kuca, K., Valko, M., 2023. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Archives of Toxicology 2023 97:10 97, 2499–2574. https://doi.org/10.1007/S00204-023-03562-9

Juan-García, A., Carbone, S., Ben-Mahmoud, M., Sagratini, G., Mañes, J., 2020. Beauvericin and ochratoxin A mycotoxins individually and combined in HepG2 cells alter lipid peroxidation, levels of reactive oxygen species and glutathione. Food and Chemical Toxicology 139, 111247. https://doi.org/10.1016/J.FCT.2020.111247

Juan-García, A., Juan, C., Manyes, L., Ruiz, M.J., 2016. Binary and tertiary combination of alternariol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol on HepG2 cells: Toxic effects and evaluation of degradation products. Toxicology in Vitro 34, 264–273. https://doi.org/10.1016/J.TIV.2016.04.016

Juan-García, A., Juan, C., Tolosa, J., Ruiz, M.J., 2019. Effects of deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol on parameters associated with oxidative stress in HepG2 cells. Mycotoxin Res 35, 197–205. https://doi.org/10.1007/S12550-019-00344-0/FIGURES/4

Juan-García, A., Taroncher, M., Font, G., Ruiz, M.J., 2018. Micronucleus induction and cell cycle alterations produced by deoxynivalenol and its acetylated derivatives in individual and combined exposure on HepG2 cells. Food and Chemical Toxicology 118, 719–725. https://doi.org/10.1016/J.FCT.2018.06.024

Juan-García, A., Tolosa, J., Juan, C., Ruiz, M.J., 2019. Cytotoxicity, Genotoxicity and Disturbance of Cell Cycle in HepG2 Cells Exposed to OTA and BEA: Single and Combined Actions. Toxins 2019, Vol. 11, Page 341 11, 341. https://doi.org/10.3390/TOXINS11060341

Juan-García, A.; Manyes, L.; Ruiz, M.J.; Font, G. Involvement of Enniatins-Induced Cytotoxicity in Human HepG2 Cells. Toxicol Lett 2013, 218, 166–173, doi:10.1016/J.TOXLET.2013.01.014.

Juan-García, A.; Ruiz, M.J.; Font, G.; Manyes, L. Enniatin A1, Enniatin B1 and Beauvericin on HepG2: Evaluation of Toxic Effects. Food and Chemical Toxicology 2015, 84, 188–196, doi:10.1016/J.FCT.2015.08.030.

Jurado, M., Vázquez, C., Patiño, B., González-Jaén, M.T., 2005. PCR detection assays for the trichothecene-producing species Fusarium graminearum, Fusarium culmorum, Fusarium poae, Fusarium equiseti and Fusarium sporotrichioides. Syst Appl Microbiol 28, 562–568. https://doi.org/10.1016/J.SYAPM.2005.02.003

Kabak, B., 2009. The fate of mycotoxins during thermal food processing. J Sci Food Agric 89, 549–554. https://doi.org/10.1002/JSFA.3491

Kalinina, S.A.; Jagels, A.; Cramer, B.; Geisen, R.; Humpf, H.U. Influence of Environmental Factors on the Production of Penitrems A–F by Penicillium Crustosum. Toxins (Basel) 2017, 9, doi:10.3390/TOXINS9070210.

Kerr, I. BCRP-Breast Cancer Resistance Protein. xPharm: The Comprehensive Pharmacology Reference 2009, 1–11, doi:10.1016/B978-008055232-3.64033-4.

Kirkland, D., Reeve, L., Gatehouse, D., Vanparys, P., 2011. A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. Mutat Res 721, 27–73. https://doi.org/10.1016/J.MRGENTOX.2010.12.015

Kirsch-Volders, M., Plas, G., Elhajouji, A., Lukamowicz, M., Gonzalez, L., Vande Loock, K., Decordier, I., 2011. The in vitro MN assay in 2011: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance. Archives of Toxicology 2011 85:8 85, 873–899. https://doi.org/10.1007/S00204-011-0691-4

Klaunig, J.E. Acrylamide Carcinogenicity. J Agric Food Chem 2008, 56, 5984–5988, doi:10.1021/JF8004492.

Kouadio, J.H., Dano, S.D., Moukha, S., Mobio, T.A., Creppy, E.E., 2007. Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon 49, 306–317. https://doi.org/10.1016/J.TOXICON.2006.09.029

Kulcsár, S., Turbók, J., Kövér, G., Balogh, K., Zándoki, E., Ali, O., Szabó, A., Mézes, M., 2024. Exposure to a Combination of Fusarium Mycotoxins Leads to Lipid Peroxidation and Influences Antioxidant Defenses, Fatty Acid Composition of Phospholipids, and Renal Histology in Laying Hens. Toxins (Basel) 16. https://doi.org/10.3390/TOXINS16050226

Lagunas-Rangel, F.A., Linnea-Niemi, J.V., Kudłak, B., Williams, M.J., Jönsson, J., Schiöth, H.B., 2022. Role of the Synergistic Interactions of Environmental Pollutants in the Development of Cancer. Geohealth 6. https://doi.org/10.1029/2021GH000552

Lakshmi, D., Gopinath, K., Jayanthy, G., Anjum, S., Prakash, D., Sudhandiran, G., 2012. Ameliorating effect of fish oil on acrylamide induced oxidative stress and neuronal apoptosis in cerebral cortex. Neurochem Res 37, 1859–1867. https://doi.org/10.1007/S11064-012-0794-1/FIGURES/5

Lapin, E.P.; Maker, H.S.; Weissbarth, S.; Weiss, C.; Lehrer, G.M. The Influence of Systemic Factors on Acrylamide-Induced Changes in Brain, Nerve, and Other Tissues. J Neurosci Res 1984, 11, 395–404, doi:10.1002/JNR.490110407.

Lei, M., Zhang, N., Qi, D., 2013. In vitro investigation of individual and combined cytotoxic effects of aflatoxin B1 and other selected mycotoxins on the cell line porcine

kidney 15. Experimental and Toxicologic Pathology 65, 1149–1157. https://doi.org/10.1016/J.ETP.2013.05.007

Lin, Y.; Wang, X. Tumor Necrosis Factor and Cancer, Buddies or Foes? 1. Acta Pharmacol Sin 2008, 29, 1275–1288, doi:10.1111/j.1745-7254.2008.00889.x.

List of Classifications – IARC Monographs on the Identification of Carcinogenic Hazards to Humans Available online: https://monographs.iarc.who.int/list-of-classifications/ (accessed on 11 April 2023).

Liu, M.; Zhao, L.; Gong, G.; Zhang, L.; Shi, L.; Dai, J.; Han, Y.; Wu, Y.; Khalil, M.M.; Sun, L. Invited Review: Remediation Strategies for Mycotoxin Control in Feed. Journal of Animal Science and Biotechnology 2022 13:1 2022, 13, 1–16, doi:10.1186/S40104-021-00661-4.

Lopez-Suarez, L., Awabdh, S. Al, Coumoul, X., Chauvet, C., 2022. The SH-SY5Y human neuroblastoma cell line, a relevant in vitro cell model for investigating neurotoxicology in human: Focus on organic pollutants. Neurotoxicology 92, 131–155. https://doi.org/10.1016/J.NEURO.2022.07.008

Mahadevan, B., Snyder, R.D., Waters, M.D., Benz, R.D., Kemper, R.A., Tice, R.R., Richard, A.M., 2011. Genetic Toxicology in the 21st Century: Reflections and Future Directions. Environ Mol Mutagen 52, 339. https://doi.org/10.1002/EM.20653

Menz, J., Götz, M.E., Gündel, U., Gürtler, R., Herrmann, K., Hessel-Pras, S., Kneuer, C., Kolrep, F., Nitzsche, D., Pabel, U., Sachse, B., Schmeisser, S., Schumacher, D.M., Schwerdtle, T., Tralau, T., Zellmer, S., Schäfer, B., 2023. Genotoxicity assessment: opportunities, challenges and perspectives for quantitative evaluations of dose–response data. Arch Toxicol 97, 2303. https://doi.org/10.1007/S00204-023-03553-W

Mesias, M.; Delgado-Andrade, C.; Morales, F.J. An Updated View of Acrylamide in Cereal Products. 2022, doi:10.1016/j.cofs.2022.100847.

Mézes, M., Kovács, M., Szabó, A., 2021. Mycotoxin exposure, oxidative stress, and lipid peroxidation. Toxicology: Oxidative Stress and Dietary Antioxidants 191–200. https://doi.org/10.1016/B978-0-12-819092-0.00020-0

Milkovic, L., Gasparovic, A.C., Cindric, M., Mouthuy, P.A., Zarkovic, N., 2019. Short Overview of ROS as Cell Function Regulators and Their Implications in Therapy Concepts. Cells 8. https://doi.org/10.3390/CELLS8080793

Mitchell, C.T., Bridgeman, L., Moyano-López, C., Penalva-Olcina, R., Juan, C., Juan-García, A., 2024. Study of cytotoxicity in neuroblastoma cell line exposed to patulin and citrinin. Food Chem Toxicol 186. https://doi.org/10.1016/J.FCT.2024.114556

Moldes-Anaya, A.; Wilkins, A.L.; Rundberget, T.; Faeste, C.K. In Vitro and in Vivo Hepatic Metabolism of the Fungal Neurotoxin Penitrem A. Drug Chem Toxicol 2009, 32, 26–37, doi:10.1080/01480540802416232.

Moldes-Anaya, A.S.; Fonnum, F.; Eriksen, G.S.; Rundberget, T.; Walaas, S.I.; Wigestrand, M.B. In Vitro Neuropharmacological Evaluation of Penitrem-Induced Tremorgenic Syndromes: Importance of the GABAergic System. Neurochem Int 2011, 59, 1074–1081, doi:10.1016/J.NEUINT.2011.08.014.

More, S., Bampidis, V., Benford, D., Boesten, J., Bragard, C., Halldorsson, T., Hernandez-Jerez, A., Hougaard-Bennekou, S., Koutsoumanis, K., Naegeli, H., Nielsen, S.S., Schrenk, D., Silano, V., Turck, D., Younes, M., Aquilina, G., Crebelli, R., Gürtler, R., Hirsch-Ernst, K.I., Mosesso, P., Nielsen, E., Solecki, R., Carfı, M., Martino, C., Maurici, D., Morte, J.P., Schlatter, J., 2019. Genotoxicity assessment of chemical mixtures. EFSA Journal 17, e05519. https://doi.org/10.2903/J.EFSA.2019.5519

Navruz-Varlı, S., Mortaş, H., 2024. Acrylamide formation in air-fried versus deep and oven-fried potatoes. Front Nutr 10. https://doi.org/10.3389/FNUT.2023.1297069

Nikolic, V.; Göncu, N.; Tas, lu; Kocadag, T.; Simic, M.; Gökmen, V. Acrylamide in Corn-Based Thermally Processed Foods: A Review. Cite This: J. Agric. Food Chem 2022, 2022, 4165–4181, doi:10.1021/acs.jafc.1c07249.

OECD (2020), *Test No. 471: Bacterial Reverse Mutation Test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071247-en.

OECD (2023), *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264861-en.

Okuno, T.; Matsuoka, M.; Sumizawa, T.; Igisu, H. Involvement of the Extracellular Signal-Regulated Protein Kinase Pathway in Phosphorylation of P53 Protein and Exerting Cytotoxicity in Human Neuroblastoma Cells (SH-SY5Y) Exposed to Acrylamide. Arch Toxicol 2006, 80, 146–153, doi:10.1007/S00204-005-0022-8.

Pan, X., Wu, X., Yan, D., Peng, C., Rao, C., Yan, H., 2018. Acrylamide-induced oxidative stress and inflammatory response are alleviated by N-acetylcysteine in PC12 cells: Involvement of the crosstalk between Nrf2 and NF-κB pathways regulated by MAPKs. Toxicol Lett 288, 55–64. https://doi.org/10.1016/J.TOXLET.2018.02.002

Patterson, D.S., Roberts, B.A., Shreeve, B.J., MacDonald, S.M., Hayes, A.W., 1979. Tremorgenic toxins produced by soil fungi. Appl Environ Microbiol 37, 172–173. https://doi.org/10.1128/AEM.37.1.172-173.1979

Penalva-Olcina, R., Juan, C., Fernández-Franzón, M., Juan-García, A., 2023. Cell cycle and enzymatic activity alterations induced by ROS production in human neuroblastoma cells SH-SY5Y exposed to Fumonisin B1, Ochratoxin A and their combination. Toxicology in Vitro 93, 105670. https://doi.org/10.1016/J.TIV.2023.105670

Penalva-Olcina, R., Juan, C., Fernández-Franzón, M., Juan-García, A., 2022. Effectiveness of beetroot extract in SH-SY5Y neuronal cell protection against Fumonisin B1, Ochratoxin A and its combination. Food and Chemical Toxicology 165, 113164. https://doi.org/10.1016/J.FCT.2022.113164

Pifferi, A., Chiaino, E., Fernandez-Abascal, J., Bannon, A.C., Davey, G.P., Frosini, M., Valoti, M., 2024. Exploring the Regulation of Cytochrome P450 in SH-SY5Y Cells: Implications for the Onset of Neurodegenerative Diseases. Int J Mol Sci 25, 7439. https://doi.org/10.3390/IJMS25137439/S1

Pinton, P., Tsybulskyy, D., Lucioli, J., Laffitte, J., Callu, P., Lyazhri, F., Grosjean, F., Bracarense, A.P., Kolf-clauw, M., Oswald, I.P., 2012. Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: Differential effects on morphology, barrier ffnction, tight junction proteins, and mitogen-activated protein kinases. Toxicological Sciences 130, 180–190. https://doi.org/10.1093/TOXSCI/KFS239

Pyo, M.C., Shin, H.S., Jeon, G.Y., Lee, K.W., 2020. Synergistic Interaction of Ochratoxin A and Acrylamide Toxins in Human Kidney and Liver Cells. Biol Pharm Bull 43, 1346–1355. https://doi.org/10.1248/BPB.B20-00282

Rabindran, S.K.; He, H.; Singh, M.; Brown, E.; Collins, K.I.; Annable, A.; Greenberger, L.M. Reversal of a Novel Multidrug Resistance Mechanism in Human Colon Carcinoma Cells by Fumitremorgin C. Cancer Res 1998, 58, 5850–5858.

Reed, T.T., 2011. Lipid peroxidation and neurodegenerative disease. Free Radic Biol Med 51, 1302–1319. https://doi.org/10.1016/J.FREERADBIOMED.2011.06.027

Ren, N., Atyah, M., Chen, W.Y., Zhou, C.H., 2017. The various aspects of genetic and epigenetic toxicology: testing methods and clinical applications. J Transl Med 15, 110. https://doi.org/10.1186/S12967-017-1218-4

Riboldi, B.P., Vinhas, Á.M., Moreira, J.D., 2014. Risks of dietary acrylamide exposure: A systematic review. Food Chem 157, 310–322. https://doi.org/10.1016/J.FOODCHEM.2014.02.046

Richard, J.L.; Peden, W.M.; Thurston, J.R. Occurrence of Penitrem Mycotoxins and Clinical Manifestations of Penitrem Intoxications. Diagnosis of Mycotoxicoses 1986, 51–59, doi:10.1007/978-94-009-4235-6_5.

Rumora, L., Kovačić, S., Rozgaj, R., Čepelak, I., Pepeljnjak, S., Žanić Grubišić, T., 2002. Cytotoxic and genotoxic effects of fumonisin B1 on rabbit kidney RK13 cell line. Arch Toxicol 76, 55–61. https://doi.org/10.1007/S00204-001-0304-8/METRICS

Rundberget, T., Skaar, I., Flåøyen, A., n.d. The presence of Penicillium and Penicillium mycotoxins in food wastes. https://doi.org/10.1016/S0168-1605(03)00291-5

Sabater-Vilar, M., Nijmeijer, S., Fink-Gremmels, J., 2003. Genotoxicity Assessment of Five Tremorgenic Mycotoxins (Fumitremorgen B, Paxilline, Penitrem A, Verruculogen, and Verrucosidin) Produced by Molds Isolated from Fermented Meats. J Food Prot 66, 2123–2129. https://doi.org/10.4315/0362-028X-66.11.2123

Sanz-Serrano, J., Vettorazzi, A., Muruzabal, D., López de Cerain, A., Azqueta, A., 2021. In vitro genotoxicity assessment of functional ingredients: DHA, rutin and α-

tocopherol. Food and Chemical Toxicology 153, 112237. https://doi.org/10.1016/J.FCT.2021.112237

Sarion, C.; Codină, G.G.; Dabija, A. Acrylamide in Bakery Products: A Review on Health Risks, Legal Regulations and Strategies to Reduce Its Formation. Int J Environ Res Public Health 2021, 18, doi:10.3390/IJERPH18084332.

Sebastià, A., Pallarés, N., Bridgeman, L., Juan-García, A., Castagnini, J.M., Ferrer, E., Barba, F.J., Berrada, H., 2023. A critical review of acrylamide green extraction and determination in food matrices: Current insights and future perspectives. TrAC Trends in Analytical Chemistry 167, 117267. https://doi.org/10.1016/J.TRAC.2023.117267

Singh, S., Banerjee, S., Chattopadhyay, P., Borthakur, S.K., Veer, V., 2015. Deoxynivalenol induces cytotoxicity and genotoxicity in animal primary cell culture. Toxicol Mech Methods 25, 184–191. https://doi.org/10.3109/15376516.2015.1006743

Singto, T., Tassaneeyakul, W., Porasuphatana, S., 2020. Protective Effects of Purple Waxy Corn on Aflatoxin B1-induced Oxidative Stress and Micronucleus in HepG2 Cells. Indian J Pharm Sci 82, 506–513. https://doi.org/10.36468/PHARMACEUTICAL-SCIENCES.674

Šmerák, P., Bárta, I., Polívková, Z., Bártová, J., Sedmíková, M., 2001. Mutagenic effects of selected trichothecene mycotoxins and their combinations with aflatoxin B1. https://cjfs.agriculturejournals.cz/doi/10.17221/6583-CJFS.html 19, 90–96. https://doi.org/10.17221/6583-CJFS

Song, E., Xia, X., Su, C., Dong, W., Xian, Y., Wang, W., Song, Y., 2014. Hepatotoxicity and genotoxicity of patulin in mice, and its modulation by green tea polyphenols administration. Food and Chemical Toxicology 71, 122–127. https://doi.org/10.1016/J.FCT.2014.06.009

Song, G.; Liu, Z.; Wang, L.; Shi, R.; Chu, C.; Xiang, M.; Tian, Q.; Liu, X. Protective Effects of Lipoic Acid against Acrylamide-Induced Neurotoxicity: Involvement of Mitochondrial Energy Metabolism and Autophagy. Food Funct 2017, 8, 4657–4667, doi:10.1039/C7FO01429E.

Spencer, P.S.; Schaumburg, H.H. A Review of Acrylamide Neurotoxicity. Part II. Experimental Animal Neurotoxicity and Pathologic Mechanisms. Can J Neurol Sci 1974, 1, 152–169, doi:10.1017/S0317167100119201.

Srivastava, S.P., Das, M., Seth, P.K., 1983. Enhancement of lipid peroxidation in rat liver on acute exposure to styrene and acrylamide a consequence of glutathione depletion. Chem Biol Interact 45, 373–380. https://doi.org/10.1016/0009-2797(83)90083-2

Su, D., Lu, J ., Nie, C., Guo, Z., Li, C., Yu, Q., Xie, J., Chen, Y., Khaneghah, M., Su, Dan, Lu, Jiawen, Nie, Chunchao, Guo, Ziyan, Li, Chang, Yu, Qiang, Xie, Jianhua, Chen, Yi, 2023. Combined Effects of Acrylamide and Ochratoxin A on the Intestinal Barrier in Caco-2 Cells. Foods 2023, Vol. 12, Page 1318 12, 1318. https://doi.org/10.3390/FOODS12061318

Sumizawa, T.; Igisu, H. Apoptosis Induced by Acrylamide in SH-SY5Y Cells. Arch Toxicol 2007, 81, 279–282, doi:10.1007/S00204-006-0145-6.

Sumizawa, T.; Igisu, H. Release of Heat Shock Proteins from Human Neuroblastoma Cells Exposed to Acrylamide. J Toxicol Sci 2008, 33, 117–122, doi:10.2131/JTS.33.117.

Sumizawa, T.; Igisu, H. Suppression of Acrylamide Toxicity by Carboxyfullerene in Human Neuroblastoma Cells in Vitro. Arch Toxicol 2009, 83, 817–824, doi:10.1007/S00204-009-0438-7.

Taroncher, M., Fuentes, C., Rodríguez-Carrasco, Y., Ruiz, M.J., 2024. Assessment of the genotoxic and mutagenic effects induced by T-2 mycotoxin in HepG2 cells. Toxicology 501, 153712. https://doi.org/10.1016/J.TOX.2023.153712

Test No. 487: In Vitro Mammalian Cell Micronucleus Test. (2010). Test No. 487: In Vitro Mammalian Cell Micronucleus Test. https://doi.org/10.1787/9789264091016-EN

Thomas, D.N., Wills, J.W., Tracey, H., Baldwin, S.J., Burman, M., Williams, A.N., Harte, D.S.G., Buckley, R.A., Lynch, A.M., 2023. Ames test study designs for nitrosamine mutagenicity testing: qualitative and quantitative analysis of key assay parameters. Mutagenesis 39, 78. https://doi.org/10.1093/MUTAGE/GEAD033

Titenko-Holland, N., Ahlborn, T., Lowe, X., Shang, N., Smith, M.T., Wyrobek, A.J., 1998. Micronuclei and developmental abnormalities in 4-day mouse embryos after paternal treatment with acrylamide. Environ Mol Mutagen 31, 206–217. https://doi.org/10.1002/(SICI)1098-2280(1998)31:3

Tournas, V. Heat-Resistant Fungi of Importance to the Food and Beverage Industry. http://dx.doi.org/10.3109/10408419409113558 2008, 20, 243–263, doi:10.3109/10408419409113558.

Ülger, T.G., Uçar, A., Çakıroğlu, F.P., Yilmaz, S., 2020. Genotoxic effects of mycotoxins. Toxicon 185, 104–113. https://doi.org/10.1016/J.TOXICON.2020.07.004

Walter, S.L. Acute Penitrem A and Roquefortine Poisoning in a Dog. Can Vet J 2002, 43, 372–374.

Wang, J.S., Groopman, J.D., 1999. DNA damage by mycotoxins. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 424, 167–181. https://doi.org/10.1016/S0027-5107(99)00017-2

Wehner, F.C., Marasas, W.F.O., Thiel, P.G., 1978. Lack of mutagenicity to Salmonella typhimurium of some Fusarium mycotoxins. Appl Environ Microbiol 35, 659. https://doi.org/10.1128/AEM.35.4.659-662.1978

Yan, D.; Pan, X.; Yao, J.; Wang, D.; Wu, X.; Chen, X.; Shi, N.; Yan, H. MAPKs and NF-KB-Mediated Acrylamide-Induced Neuropathy in Rat Striatum and Human Neuroblastoma Cells SY5Y. J Cell Biochem 2019, 120, 3898–3910, doi:10.1002/JCB.27671.

Yan, D.; Wang, N.; Yao, J.; Wu, X.; Yuan, J.; Yan, H. Curcumin Attenuates the PERK-EIF2α Signaling to Relieve Acrylamide-Induced Neurotoxicity in SH SY5Y Neuroblastoma Cells. Neurochem Res 2022, 47, 1037–1048, doi:10.1007/S11064-021-03504-W.

Ye, Y., Wei, K., Ji, J., Ning, X., Wang, J.S., Sun, X., 2023. Toxicity and metabolic effect of combined deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol on a Caco-2 cell model. Food Biosci 53, 102718. https://doi.org/10.1016/J.FBIO.2023.102718

Yousef, M.I., El-Demerdash, F.M., 2006. Acrylamide-induced oxidative stress and biochemical perturbations in rats. Toxicology 219, 133–141. https://doi.org/10.1016/J.TOX.2005.11.008

Yuan, Y.; Chen, K.; Chen, X.; Wang, C.; Qiu, H.; Cao, Z.; Song, D.; Sun, Y.; Guo, J.; Tickner, J.; et al. Fumitremorgin C Attenuates Osteoclast Formation and Function via Suppressing RANKL-Induced Signaling Pathways. Front Pharmacol 2020, 11, 238, doi:10.3389/FPHAR.2020.00238/BIBTEX.

Zamani, E., Shokrzadeh, M., Modanloo, M., Shaki, F., 2018. In Vitro Study Towards Role of Acrylamide-Induced Genotoxicity in Human Lymphocytes and the Protective

Effect of L- Carnitine. Brazilian Archives of Biology and Technology 61, e18160685. https://doi.org/10.1590/1678-4324-20181600685

Zhang, Y., Ouyang, B., Zhang, W., Guang, C., Xu, W., Mu, W., 2024. Deoxynivalenol: Occurrence, toxicity, and degradation. Food Control 155, 110027. https://doi.org/10.1016/J.FOODCONT.2023.110027

Zhao, L.; Zhang, L.; Xu, Z.; Liu, X.; Chen, L.; Dai, J.; Karrow, N.A.; Sun, L. Occurrence of Aflatoxin B 1 , Deoxynivalenol and Zearalenone in Feeds in China during 2018-2020., doi:10.1186/s40104-021-00603-0.

Zhao, M., Deng, L., Lu, X., Fan, L., Zhu, Y., Zhao, L., 2022. The involvement of oxidative stress, neuronal lesions, neurotransmission impairment, and neuroinflammation in acrylamide-induced neurotoxicity in C57/BL6 mice. Environmental Science and Pollution Research 29, 41151–41167. https://doi.org/10.1007/S11356-021-18146-2/FIGURES/7

Zhao, M., Lewis Wang, F.S., Hu, X., Chen, F., Chan, H.M., 2017. Acrylamide-induced neurotoxicity in primary astrocytes and microglia: Roles of the Nrf2-ARE and NF-κB pathways. Food and Chemical Toxicology 106, 25–35. https://doi.org/10.1016/J.FCT.2017.05.007

Zhou, S. min, Jiang, L. ping, Geng, C. yan, Cao, J., Zhong, L. fu, 2009. Patulin-induced genotoxicity and modulation of glutathione in HepG2 cells. Toxicon 53, 584–586. https://doi.org/10.1016/J.TOXICON.2009.01.030

Zhou, Y., Li, J., Wang, C., Pan, Z., 2022. Fumitremorgin C alleviates advanced glycation end products (AGE)-induced chondrocyte inflammation and collagen II and aggrecan degradation through sirtuin-1 (SIRT1)/nuclear factor (NF)-κB/ mitogen-

activated protein kinase (MAPK). https://doi.org/10.1080/21655979.2021.2024387 13, 3867–3876.

Zingales, V., Fernández-Franzón, M., & Ruiz, M. J. (2021). Sterigmatocystin-induced DNA damage triggers cell-cycle arrest via MAPK in human neuroblastoma cells. Toxicology Mechanisms and Methods, 31(7), 479–488. https://doi.org/10.1080/15376516.2021.1916801

ANNEX

Anexo

ANNEX I

toxins

MDPI

Review

Effect of Acrylamide and Mycotoxins in SH-SY5Y Cells: A Review

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Abstract: Thermal processes induce the formation of undesired toxic components, such as acrylamide (AA), which has been shown to induce brain toxicity in humans and classified as Group 2A by the International Agency of Research in Cancer (IARC), as well as some mycotoxins. AA and mycotoxins' toxicity is studied in several in vitro models, including the neuroblastoma cell line model SH-SY5Y cells. Both AA and mycotoxins occur together in the same food matrix cereal base (bread, pasta, potatoes, coffee roasting, etc.). Therefore, the goal of this review is to deepen the knowledge about the neurological effects that AA and mycotoxins can induce on the in vitro model SH-SY5Y and its mechanism of action (MoA) focusing on the experimental assays reported in publications of the last 10 years. The analysis of the latest publications shows that most of them are focused on cytotoxicity, apoptosis, and alteration in protein expression, while others are interested in oxidative stress, axonopathy, and the disruption of neurite outgrowth. While both AA and mycotoxins have been studied in SH-SY5Y cells separately, the mixture of them is starting to draw the interest of the scientific community. This highlights a new and interesting field to explore due to the findings reported in several publications that can be compared and the implications in human health that both could cause. In relation to the assays used, the most employed were the MTT, axonopathy, and qPCR assays. The concentration dose range studied was 0.1–10 mM for AA and 2 fM to 200 µM depending on the toxicity and time of exposure for mycotoxins. A healthy and varied diet allows the incorporation of a large family of bioactive compounds that can mitigate the toxic effects associated with contaminants present in food. Although this has been reported in some publications for mycotoxins, there is still a big gap for AA which evidences that more investigations are needed to better explore the risks for human health when exposed to AA and mycotoxins.

Keywords: acrylamide; mycotoxins; food processed contaminants; neurotoxicity; in vitro

Key Contribution: Acrylamide (AA) (0.1 to 10 mM) and mycotoxins (2 fM to 200 µM) causes toxic effects in neuroblastoma cells, SH-SY5Y. This review highlights the importance of performing studies based in the mixture of mycotoxins and food processed contaminants such as acrylamide.

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1. Introduction

The food baking process is the most important sub-process responsible for the main chemical, physical, and sensory properties of the final product, as well as the development of bioactive and antioxidant compounds [1–3]. However, this thermal process induces the formation of unwanted toxic components, including acrylamide (AA), due to the Maillard reaction occurring at high temperatures [1,4–6]. In detail, the Maillard reaction involves three major steps: (i) condensation of free amino groups (such as asparagine) with reducing sugars (glucose and fructose) to form acrolein; (ii) Strecker degradation of amino acids to aldehydes and ammonia; and (iii) brown nitrogenous compounds combining with acrylic acid to form AA (Figure 1) [7].

ANNEX II

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Individual and combined effect of acrylamide, fumitremorgin C and penitrem A on human neuroblastoma SH-SY5Y cells

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ABSTRACT

Acrylamide (AA) is a chemical compound that can be formed in certain foods during high-temperature cooking processes such as frying, baking, and roasting. Exposure to AA has been linked to several neurological effects, including peripheral neuropathy, ataxia, and impaired cognitive function. Penitrem A (PEN A) and Fumitremorgin C (FTC) are toxic mycotoxins produced by certain species of fungi, such as *Penicillium Crustosum*, *Aspergillus Fumigatus* and *Neosartorya Fischeri*. Both mycotoxins are commonly found in contaminated foods and animal feeds and have been linked to several adverse health effects in humans and animals, including the ability to disrupt normal functioning of the nervous system, tremors, seizures, muscle spasms, and convulsions. AA, PEN A, and FTC are all chemical contaminants. Understanding their toxicity and how they may affect human cells can help food safety authorities to establish safe exposure levels for these compounds through food and develop strategies to reduce their presence. The aim of this study was to explore the combined *in vitro* toxicological effects of AA, PEN A and FTC in SH-SY5Y cells. For this purpose, cells were treated with AA, FTC, and PEN A as an individual and combined treatment. The types of interactions were assessed by the isobologram analysis. The cell cycle was performed by flow cytometry. Additive effect in binary and tertiary combinations was the major effect according to isobologram graphics. Our results demonstrate that PEN A possessed the highest potential in disturbing cell cycle progression by disrupting cell density in G0/G1 phase.

1. Introduction

Acrylamdie (AA), Fumitremorgin C (FTC), and Penitrem A (PEN A) are all chemical compounds that can be found in certain foods and have been associated with potential health risks (Fig. 1). AA is formed when certain foods are cooked at high temperatures, particularly in processes such as frying, baking, or roasting (Sarion et al., 2021; Sebastià et al., 2023). Foods that are high in starch, such as potatoes, bread, and coffee, are particularly prone to AA formation.

AA has been linked to an increased risk of cancer in animal studies, although its effects on humans are still being studied (Klaunig, 2008a; IARC). AA is classified as probable carcinogen (2A) substance by the International Agency for Research on Cancer (IARC) (IARC, 1994). Exposure to AA has been linked to several neurological effects, including peripheral neuropathy, ataxia, and impaired cognitive function (Spencer and Schaumburg, 1974). Studies in animals have shown that AA can damage nerve cells and disrupt the balance of neurotransmitters in the brain, leading to various neurological symptoms (Allam et al., 2011; Klaunig, 2008b; Lapin et al., 1984). One of the mechanisms through which AA causes neurotoxicity is by forming adducts with proteins in nerve cells, which can alter their function and structure. AA can also disrupt the production of ATP, which is essential for the proper functioning of nerve cells (Erkekoglu and Baydar, 2014).

Besides, it has been demonstrated that humans can be exposed to a large number of mycotoxins through contaminated foods and feeds (Liu et al., 2022; Zhao et al., 2021), that is why on this studied we not only focused our attention on chemical contaminants, but also in two scarcely studied mycotoxins that can contaminate one of the main sources of AA (Nikolic et al., 2022). FTC and PEN A, toxic mycotoxins that are produced by certain species of fungi such as *Penicillium crustosum* and *Aspergillus fumigatus* (Kalinina et al., 2017; Yuan et al., 2020) and can contaminate some cereals such as corn (Dorner et al., 1984). Specifically, FTC is often present in contaminated cereals, grains, and hay, while PEN A is commonly found in contaminated nuts, dried fruits, and grains (Richard et al., 1986; Tournas, 2008). Both mycotoxins have been associated with adverse health effects in animals and humans, including tremors, seizures, and muscle spasms (Berntsen et al., 2017a). FTC, belonging to a class of diketopiperazines that are potent mycotoxins.

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ANNEX III

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RESEARCH ARTICLE OPEN ACCESS

Evaluating the Genotoxicity and Mutagenicity of Food Contaminants: Acrylamide, Penitrem A, and 3-Acetyldeoxynivalenol in Individual and Combined Exposure In Vitro

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Keywords: 3-acetyldeoxynivalenol genotoxicity and mutagenicity | acrylamide | penitrem A

ABSTRACT

This study aimed to evaluate the genotoxic effects of food contaminants exposure in human neuroblastoma SH-SY5Y cells using the micronucleus (MN) assay and Ames test. Acrylamide (AA), penitrem A (PEN A), and 3-acetyldeoxynivalenol (3-ADON) were tested both individually and in combination. Since humans are likely to be exposed to these substances simultaneously through diet, it is crucial to investigate their combined effects of the compounds rather than just their individual toxicities. The results demonstrated significant increases in MN frequency for all individual treatments and in a dose-dependent manner for AA and 3-ADON. Combined treatments also resulted in higher MN frequencies, particularly for AA + 3-ADON and PEN A + 3-ADON respect to the control. However, the Ames test revealed no mutagenic potential for any of the individual or combined treatments, consistent with previous studies. These findings suggest that while food contaminants induce chromosomal damage (MN induction), they do not cause gene mutations. Nonetheless, the lack of single mutations activity does not exclude the potential health risks of combined mycotoxin exposure, especially given the observed genotoxicity due to the DNA damage through chromosomal aberrations. Future studies focused on the mechanism of action should investigate the combined effects of food contaminants in more detail to better assess their potential health risks.

1 | Introduction

The importance of studying genotoxicity becomes particularly evident when investigating food processing and natural contaminants such as acrylamide (AA), penitrem A (PEN A) and 3-acetyldeosynivalenol (3-ADON) each of which possesses significant risks to human health through their genotoxic and toxic properties (Bridgeman et al. 2023; Han et al. 2014; Riboldi et al. 2014). The interest of studying effect of contaminants at neuronal level has recently increased and SH-SY5Y cells are a good model to deep into such effect (Bridgeman et al. 2024; Lopez-Suarez et al. 2022). Nevertheless, some study requires the use of other in vitro models, as it is the case of studying mutagenicity (Ames test) with different bacteria strains (OECD 2020). Altogether allows obtaining a broader spectrum of the toxicity effects that can complete the understanding the consequences of toxicity of compounds. Here, it is reported the study of mutagenicity through the Ames test and the study of genotoxicity through the MN detection in SH-SY5Y cells.