

Incidence de la multi-contamination aux mycotoxines de Fusarium sur cellules humaines : évaluation de la cytotoxicité et approche toxico-protéomique

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université de bretagne occidentale

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Incidence de la multicontamination aux mycotoxines de *Fusarium* sur cellules humaines : évaluation de la cytotoxicité et approche toxicoprotéomique **Thèse soutenue le 3 novembre 2017** devant le jury composé de :

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Stéphanie MADEC MC, Université de Brest / *Co-encadrant de thèse* « Ne jamais tenter de reproduire une première expérience réussie. » Loi de Murphy Cette thèse a été cofinancée par la région Bretagne.



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Liste des abréviations

Afla : Aflatoxine

AFB1 : Aflatoxine B1

AFM1 : Aflatoxine M1

AFSSA : Agence Française de Sécurité Sanitaire des Aliments

ANSES : Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnment et du travail

BEA : Beauvericine

CAS : Chemical Abstracts Service

CI : Concentration Inhibitrice

CIT: Citrinine

CIRC : Centre International de Recherche sur le Cancer (acronyme anglais : IARC)

CE : Commission Européenne

DAS : Diacétoxyscirpénol

DJT: Dose Journalière Tolérable

DL : Dose Létale

DOM-1 : Dé-époxy déoxynivalénol

DON : Déoxynivalénol

EAT : Etude de l'Alimentation Totale

EFSA : European Food Safety Authority (Autorité européenne de sécurité des aliments)

ENN : Enniatine

FAO: Food and Agriculture Organization of the United Nations (Organisation des Nations

Unies pour l'alimentation et l'agriculture)

FB1: Fumonisine B1

FB2: Fumonisine B2

FB3 : Fumonisine B3

FDA : Food and Drug Administration (Agence américaine des produits alimentaires et médicamenteux)

FUM : Fumonisine

FUS : Fusaproliférine

FUS-X : Fusarénone X

HT2 : Toxine HT-2

IC : Indice de Combinaison

JECFA : Joint FAO/WHO Expert Committee on Food Additives (Comité d'experts administré conjointement par le FAO et l'OMS sur les additifs alimentaires)

LC-MS/MS : Liquid Chromatography coupled to tandem Mass Spectrometry (Chromatographie liquide couplée à la spectrométrie de masse en tandem)

LOAEL: Lowest Observed Adverse Effect Level (Dose minimale avec un effet nocif observé)

MAPK: Mitogen-Activated Protein Kinase (Protéine kinase activée par des agents mitogènes)

MON: Moniliformine

MTS: Mitochondrial Tetrazolium Salt

NIV : Nivalénol

NOAEL : No Observed Adverse Effect Level (Dose sans effet néfaste observé)

NOEL: No Observed Effect Level (Dose sans effet observé)

OTA : Ochratoxine A

PAT : Patuline

ROS: Reactive Oxygen Species (Dérivés réactifs de l'oxygène)

SCF: Scientific Commitée of Food (Commité scientifique Européen de l'alimentation humaine)

T2 : Toxine T-2

TCT: Trichothécène

TEER : TransEpithelial Electrical Resistance (Résistance électrique transépithéliale)

WHO: World Health Organization (Organisation mondiale de la santé (OMS))

ZEA : Zéaralénone

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Avant-propos

Les céréales et les produits issus de leur transformation représentent la ressource alimentaire humaine et animale la plus importante au monde. Ces matrices sont néanmoins susceptibles d'être contaminées par des microorganismes, parmi lesquels certaines espèces de champignons sont toxinogènes, c'est-à-dire capables de produire des mycotoxines. La production de ces contaminants par les champignons dépend des conditions environnementales, essentiellement du climat, c'est pourquoi leur présence dans l'alimentation est difficile à prévenir et contrôler. L'Homme est ainsi exposé tout au long de sa vie à travers son alimentation à ces contaminants naturels, généralement à de faibles doses et en mélange.

De nombreux pays ont établi des réglementations ou recommandations fixant les teneurs maximales autorisées dans les aliments pour les mycotoxines les plus problématiques d'un point de vue sanitaire et économique. Cependant, à ce jour, aucune de ces réglementations ne tient compte des effets combinés des mycotoxines, pourtant couramment retrouvées en mélange dans notre alimentation, et pouvant agir de façon antagoniste, additive et, dans le pire des cas, synergique. Face au manque de données toxicologiques concernant les mélanges de mycotoxines, il devient urgent de mieux identifier les conditions (doses, mélanges, ...) et les mécanismes responsables de leur toxicité chez l'Homme. Le choix des approches expérimentales pour de telles études est donc particulièrement important, d'autant plus qu'aujourd'hui, les différences de réponse entre l'Homme et l'animal sont bien connues.

L'épithélium intestinal et le système immunitaire, qui constituent la première barrière de défense de l'hôte suite à l'ingestion de contaminants alimentaires, représentent des modèles d'étude pertinents en toxicologie. D'autre part, de par son rôle majeur dans la biotransformation des xénobiotiques, le foie constitue un organe cible pour de nombreuses classes de produits chimiques potentiellement toxiques. Ainsi, le choix de modèles cellulaires humains d'origine intestinale, immunitaire et hépatique apparaît adapté à l'évaluation de la toxicité aiguë et chronique des mycotoxines les plus présentes dans nos régions, seules et en mélange.

Chapitre I : INTRODUCTION GENERALE

Partie I : Les mycotoxines et la problématique des mélanges

1. Les mycotoxines, généralités

1.1. Définition

Certaines espèces de champignons filamenteux (également appelées moisissures) contaminant notre l'alimentation sont capables de produire des métabolites secondaires potentiellement toxiques pour notre santé, couramment appelés « mycotoxines ».

Historiquement, le terme « mycotoxine » a été inventé en 1962 à la suite d'une crise vétérinaire atypique survenue en 1960 en Angleterre, près de Londres, au cours de laquelle environ 100000 dindonneaux périrent (de Iongh et al., 1962; Wannop, 1961). Lorsque cette mystérieuse épidémie nommée « Turkey X disease » fut reliée à la consommation d'arachides contaminées avec des métabolites secondaires d'*Aspergillus flavus* (aflatoxines), il apparut alors possible aux yeux de la communauté scientifique que d'autres métabolites secondaires de moisissures pouvaient avoir des effets délétères. Dès lors, la classification « mycotoxine » fut étendue à un certain nombre de toxines fongiques déjà connues, telles que les alcaloïdes de l'ergot, certains composés qui avaient été isolés à l'origine comme antibiotiques, tels que la patuline (PAT), ainsi que de nouveaux métabolites secondaires fongiques.

Les mycotoxines sont aujourd'hui définies comme des molécules chimiques non protéiques de faible poids moléculaire, issues du métabolisme secondaire des champignons, et possédant des propriétés toxicologiques potentiellement dangereuses pour l'Homme et l'animal à des doses parfois extrêmement faibles. Cependant, bien que toutes les mycotoxines soient d'origine fongique, tous les composés toxiques produits par des champignons ne sont pas appelés mycotoxines. En effet, les métabolites fongiques toxiques pour les bactéries sont appelés antibiotiques et ceux toxiques pour les plantes sont nommés phytotoxines.

D'autre part, en raison de leur grande diversité en termes de structures chimiques, de voies de biosynthèse, de production et d'effets toxiques, les mycotoxines ne sont pas seulement difficiles à définir, elles sont également compliquées à classer. Elles peuvent ainsi être regroupées en fonction de l'organe qu'elles affectent : hépatotoxiques, œstrogéniques, immuno-/hématotoxiques, néphrotoxiques, neurotoxiques, etc ; ou dans des groupes plus génériques : génotoxiques, tératogéniques ou encore carcinogéniques. Elles peuvent également être séparées selon leur structure chimique et leur origine biologique : lactones,

polycétoacides, terpènes, cyclopeptides, métabolites azotés, dérivés d'acides aminés, etc ; ou encore selon les champignons qui les produisent : toxines d'*Aspergillus*, toxines de *Fussarium* (ou fusariotoxines), toxines de *Penicillium*, etc (Bennett and Klich, 2003).

1.2. Production

Les contaminations par les champignons filamenteux et leurs mycotoxines peuvent survenir aussi bien sur la plante au champ, qu'après la récolte, pendant le transport, lors du stockage ou lors des procédés de fabrication (Bryden, 2012). La présence de champignons filamenteux et de mycotoxines dépend essentiellement des conditions environnementales, telles que la température et l'humidité. Cependant, bien que le climat soit un des facteurs clés, d'autres facteurs comme les procédés agricoles, les dommages causés aux cultures par les insectes ou les maladies peuvent favoriser le développement des moisissures et la production de mycotoxines. C'est pourquoi la présence de mycotoxines dans l'alimentation est très difficile à prévenir et contrôler (Milani, 2013). De plus, l'évolution des pratiques agricoles constatée au début du XXI^{ème} siècle, telles que la diminution à l'échelle mondiale de l'utilisation de pesticides, et notamment de fongicides, associée au changement climatique, pourrait avoir des conséquences sur l'évolution du risque alimentaire lié à la contamination fongique et à la production de mycotoxines (Paterson and Lima, 2011).

D'un point de vue sanitaire et économique, les espèces fongiques les plus problématiques à ce jour appartiennent aux genres *Aspergillus, Fusarium* et *Penicillium,* bien qu'il en existe beaucoup d'autres capables de sécréter des mycotoxines, telles que les espèces des genres *Alternaria, Chaetomium, Claviceps, Diplodia, Myrothecium, Neotyphodium, Phoma, Phomopsis, Pithomyces* et *Stachybotrys* (AFSSA, 2009). En effet, parmi les milliers de métabolites secondaires fongiques aujourd'hui recensés (ce qui inclut plus de 400 mycotoxines), seules quelques mycotoxines dites « majeures », essentiellement produites par *Aspergillus, Fusarium* et *Penicillium* spp., sont réglementées en raison de leur présence dans l'alimentation et de leur toxicité pour la santé humaine et animale. En particulier, les aflatoxines (Afla), l'ochratoxine A (OTA), la patuline (PAT) et la citrinine (CIT), majoritairement produites par *Aspergillus* et/ou *Penicillium* spp., ainsi que les trichothécènes (TCTs) (et plus particulièrement le déoxynivalénol (DON)), les fumonisines (FUMs) et la zéaralénone (ZEA), principalement sécrétées par des espèces de *Fusarium*, sont la cible des réglementations de nombreux pays (Smith et al., 2016). D'autre part, de nombreuses espèces de *Fusarium* peuvent également produire des mycotoxines dites « émergentes », plus

récemment détectées et isolées, et dont les effets toxiques sont encore mal connus. Ces dernières sont souvent retrouvées dans l'alimentation en présence de fusariotoxines majeures. Les plus connues de ces mycotoxines émergentes sont la fusaproliférine (FUS), la beauvericine (BEA), les enniatines (ENNs) et la moniliformine (MON), mais celles-ci ne sont, à ce jour, pas encore réglementées (Jestoi, 2008; Smith et al., 2016).

En plus de ces mycotoxines aujourd'hui bien identifiées, des métabolites de mycotoxines dérivés des plantes, également appelés mycotoxines « masquées », ont été plus récemment observés comme co-contaminants importants dans les céréales. Plus précisément, il s'agit de mycotoxines conjuguées à de petites molécules telles que des monosaccharides, du glutathion ou des sulfates, principalement issues du système de détoxification des plantes. De telles modifications de la structure des mycotoxines par la plante rendent alors la détection de ces dérivés très compliquée par les techniques analytiques conventionnelles. C'est pourquoi la présence dans les aliments de ces métabolites considérés comme masqués n'est ni contrôlée, ni réglementée. Les fusariotoxines, en particulier DON, ZEA, T2, HT2 et NIV, sont les plus sujettes à une détoxification par les plantes. En effet, les mycotoxines masquées les plus rencontrées sont les conjugués glucose des TCTs et de la ZEA, tels que DON3Glc, NIV3Glc, HT2Glc ou encore ZEA14Glc. Les données de la littérature sur l'occurrence de ces mycotoxines masquées dans l'alimenation, mais également sur leur devenir dans l'intestin et sur leur toxicité, sont assez limitées, essentiellement en raison de la faible disponibilité de composés purifiés. Bien qu'une toxicité plus faible ait pu être observée pour les dérivés les plus étudiés (tels que DON3Glc et ZEA14Glc) par rapport aux composés parents, une biodisponibilité potentiellement importante lors de la digestion pourrait représenter une menace pour la santé du consommateur. En particulier, l'hydrolyse possible des mycotoxines masquées pour revenir sous leurs formes parentales toxiques lors de la digestion suscite quelques inquiétudes. De plus, aucune étude n'a encore été effectuée sur l'hydrolyse intestinale, l'absorption ou encore sur la toxicité de mycotoxines conjuguées à des oligosaccharides plus complexes (Berthiller et al., 2013; Gratz, 2017).

1.3. Occurrence

Ces contaminants naturels de l'alimentation sont retrouvés partout à travers le monde dans les denrées d'origine végétale, essentiellement dans les céréales, mais aussi dans les fourrages, les fruits, les légumes, les noix, les graines de café et de cacao, les épices et les produits transformés. Certaines mycotoxines ou leurs métabolites sont également retrouvés dans les

produits d'origine animale tels que le lait, les œufs et la viande, suite à l'ingestion de rations contaminées par les animaux (Bryden, 2012; Marin et al., 2013). De plus, les mycotoxines sont peu ou pas éliminées lors des différentes étapes de transformation des produits alimentaires, c'est pourquoi on les retrouve également dans les produits transformés (Bullerman and Bianchini, 2007).

D'autre part, aucune zone du globe ne semble être épargnée par la présence de mycotoxines (BIOMIN, 2016; Rodrigues and Naehrer, 2012). Cependant, les conditions climatiques et les pratiques agricoles spécifiques à chaque région peuvent favoriser la production de certaines mycotoxines par rapport à d'autres. C'est le cas, par exemple, des aflatoxines que l'on retrouve essentiellement en Afrique et en Asie. Dans les zones tempérées du globe, telles que l'Europe ou l'Amérique du Nord, les mycotoxines les plus retrouvées sont des fusariotoxines (Creppy, 2002; Smith et al., 2016). Toutefois, l'importation de denrées contaminées favorise la dispersion de ces mycotoxines à travers le monde.

1.4. Exposition

Certaines mycotoxines ont une toxicité aiguë très marquée, provoquant des « mycotoxicoses » aiguës. Historiquement, la mycotoxicose humaine la plus anciennement connue est l'ergotisme, également connue sous le nom de « mal des ardents » ou « feu Saint-Antoine », provoquée par l'ingestion des toxines de *Claviceps purpurea* (ergot du seigle). Des épidémies d'ergotisme ont sévi du VIII^{ème} au XVI^{ème} siècle en raison des conditions d'alimentation difficiles des populations, provoquant des délires, de violentes douleurs, des abcès et des gangrènes des extrémités pouvant aboutir à des infirmités graves. Cependant, aujourd'hui en Europe, il est exceptionnel d'être exposé à de très fortes doses de mycotoxines après une seule ingestion d'aliments contaminés (AFSSA, 2009). Les effets d'une exposition répétée à de faibles doses (exposition chronique) sont les plus redoutés en raison des habitudes alimentaires du consommateur et du pouvoir de rémanence de ces toxines. Cependant, les données toxicologiques à partir d'études menées dans des conditions d'exposition chronique aux mycotoxines.

L'exposition des populations aux mycotoxines présentes dans les aliments est surveillée à travers les études de l'alimentation totale (EAT), réalisées à l'échelle nationale dans de nombreux pays. Ces études reposent sur une méthodologie standardisée et recommandée par

l'organisation mondiale de la santé (OMS). Les EAT consistent à prélever sur différents points de vente les aliments régulièrement consommés par la population et à les préparer tels qu'ils sont consommés avant de les analyser. En France, l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnment et du travail (ANSES) a réalisé une première étude (EAT1) entre 2000 et 2004 en collaboration avec l'Institut national de recherche agronomique (INRA) (Leblanc et al., 2005), puis une seconde étude (EAT2) entre 2006 et 2009 (Sirot et al., 2013). Ces études concernaient une douzaine de mycotoxines, incluant les mycotoxines majeures, ainsi que certains de leurs dérivés (tels que 3-ADON et 15-ADON, qui sont des composés acétylés du DON). De façon générale, il est difficile de comparer les résultats de l'EAT1 et de l'EAT2 concernant ces mycotoxines en raison des différences de limites analytiques de détection et de quantification (LOD et LOQ respectivement). De plus, l'échantillonnage étant différent entre ces deux études, il n'est pas possible de comparer les moyennes de contamination entre les groupes d'aliments. Néanmoins, pour certaines mycotoxines (telles que OTA, PAT, ZEA et nivalénol -NIV-), l'exposition de la population estimée lors de l'EAT1 semble diminuer au cours de l'EAT2, alors que celle au DON paraît augmenter et celle aux Afla et aux FUMs est équivalente. Les conditions climatiques favorables ou défavorables à la production de ces toxines avant la période d'échantillonage pourraient en partie expliquer les variations d'exposition entre les deux études. Par ailleurs, ces EAT soulignent que le risque peut être écarté pour la population générale concernant la plupart des mycotoxines étudiées (Afla, OTA, PAT, FUMs, NIV et ZEA), mais pas concernant le DON et ses dérivés acétylés pour lesquels les calculs d'exposition montrent des dépassements des valeurs toxicologiques de référence.

D'autre part, grâce au développement de méthodes de détection de plus en plus perfectionnées et résolutives au cours des dernières années, il a été possible de mettre en évidence la multiexposition des Hommes et des animaux aux mycotoxines *via* notamment leur alimentation (Streit et al., 2013; Varga et al., 2013). A ce titre, la société BIOMIN qui publie un rapport annuel sur les risques liés à la contamination des aliments destinés à l'alimentation animale par les mycotoxines depuis une dizaine d'années a montré qu'en 2016 environ deux tiers des échantillons céréaliers dans le monde étaient contaminés par aux moins deux mycotoxines (BIOMIN, 2016). De nombreuses raisons peuvent expliquer cette concomitance, comme par exemple la capacité pour un même champignon à produire plusieurs mycotoxines, la contamination simultanée ou successive des aliments par plusieurs champignons, ou encore la fabrication d'aliments à partir de différentes matrices alimentaires. Malheureusement, la toxicité combinée des mycotoxines ne peut pas toujours être estimée à partir des effets toxicologiques propres à chaque mycotoxine du mélange. De plus, un mélange de mycotoxines peut présenter des effets indésirables potentiellement plus élevés que ceux observés après l'exposition à une seule mycotoxine. En effet, la multi-exposition aux mycotoxines peut entraîner des effets antagonistes, additifs ou, dans le pire des cas, synergiques (Grenier and Oswald, 2011; Smith et al., 2016). Aujourd'hui, les données sur les effets toxicologiques combinés des mycotoxines, aussi bien dans des conditions d'exposition aiguës que chroniques, sont encore limitées, et les mécanismes cellulaires de réponse à l'exposition ou la co-exposition à ces mycotoxines sont mal connus. Ainsi, le risque sanitaire lié à la multi-contamination aux mycotoxines dans l'alimentation est encore mal défini.

2. La problématique des mélanges de mycotoxines

La revue de littérature intitulée « **Natural co-occurrence of mycotoxins in foods and feeds and their** *in vitro* **combined toxicological effects** » (Smith, M-C.; Madec, S.; Coton, E. and Hymery, N. *Toxins* 2016, 8(4), 94; doi : 10.3390/toxins8040094) a été rédigée dans le but de mieux appréhender la problématique liée à la multi-contamination des aliments aux mycotoxines à l'échelle mondiale.

Dans cette revue, sont présentées les principales mycotoxines retrouvées dans l'alimentation et réglementées dans la plupart des pays. En particulier, les valeurs réglementaires et recommandations établies par l'Europe et les Etats-Unis dans les denrées alimentaires ont été comparées. De plus, cette étude met en évidence les principaux mélanges de mycotoxines retrouvés dans les aliments en s'appuyant sur plus de 100 articles scientifiques, ainsi que les combinaisons de mycotoxines les plus étudiées sur des modèles *in vitro* en s'intéressant tout particulièrement aux effets cytotoxiques combinés des fusariotoxines.



Review



Natural Co-Occurrence of Mycotoxins in Foods and Feeds and Their *in vitro* Combined Toxicological Effects

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Abstract: Some foods and feeds are often contaminated by numerous mycotoxins, but most studies have focused on the occurrence and toxicology of a single mycotoxin. Regulations throughout the world do not consider the combined effects of mycotoxins. However, several surveys have reported the natural co-occurrence of mycotoxins from all over the world. Most of the published data has concerned the major mycotoxins aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), fumonisins (FUM) and trichothecenes (TCTs), especially deoxynivalenol (DON). Concerning cereals and derived cereal product samples, among the 127 mycotoxin combinations described in the literature, AFs+FUM, DON+ZEA, AFs+OTA, and FUM+ZEA are the most observed. However, only a few studies specified the number of co-occurring mycotoxins with the percentage of the co-contaminated samples, as well as the main combinations found. Studies of mycotoxin combination toxicity showed antagonist, additive or synergic effects depending on the tested species, cell model or mixture, and were not necessarily time- or dose-dependent. This review summarizes the findings on mycotoxins and their co-occurrence in various foods and feeds from all over the world as well as *in vitro* experimental data on their combined toxicity.

Keywords: mycotoxins; foodstuffs; regulations; co-occurrence; combined toxicological effects

1. Introduction

Mycotoxins are secondary fungal metabolites mainly produced by species from the *Aspergillus*, *Penicillium*, and *Fusarium* genera. These toxins are found all around the world as natural contaminants in numerous commodities of plant origin, especially in cereals grains, but also in nuts, oilseeds, fruits, dried fruits, vegetables, cocoa and coffee beans, wine, beer, as well as herbs and spices. Mycotoxins can also be found in animal-derived food if animals eat contaminated feed, namely meat, eggs, milk, and milk derivatives [1,2].

Mycotoxin production, especially on grains, is highly dependent on pre and/or postharvest environmental factors (e.g., temperature and moisture content). Climate represents the key factor in mycotoxin and fungal occurrence. Mycotoxins are climate-dependent compounds but several factors can affect their presence, such as bioavailability of micronutrients, insect damage making it a complex and multifactor phenomenon [3]. These metabolites are usually subdivided into field mycotoxins, produced on cereal crops before or immediately after harvest mainly by *Fusarium* spp., and storage mycotoxins, primarily secreted by *Aspergillus* and *Penicillium* spp. during commodity drying and storage [1].

Mycotoxin ingestion may induce various chronic and acute effects on humans and animals, such as hepatotoxic, genotoxic, immunosuppressive, estrogenic, nephrotoxic, teratogenic, and/or

carcinogenic effects [1,4]. Moreover, mycotoxins are not completely eliminated during food processing operations and can contaminate finished processed food products [5,6].

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Their worldwide occurrence in various food and feeds poses a major risk for human and animal health and, as a consequence, causes economic losses [1]. Although these economic costs are impossible to estimate accurately, the United States Food and Drug Administration (FDA) evaluated, based on computer modeling, that in the USA the potential economic costs of crop losses due to mycotoxin contaminations average \$932 million per year [7]. The FAO (Food and Agriculture Organization of the United Nations) estimated that 25% of the world's crops are affected by mycotoxins each year, with annual losses of around 1 billion metric tons of food and food products (2007).

Among the thousands of fungal secondary metabolites currently known, only a few groups of mycotoxins are important from the safety and economic points of view; namely aflatoxins (AFs), mainly produced by *Aspergillus* species; ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium* species, and zearalenone (ZEA), fumonisins (FUM) and trichothecenes (TCTs) (especially deoxynivalenol (DON)), primarily produced by many *Fusarium* species [8–10]. Moreover, several species from the *Fusarium* genus can produce other mycotoxins with toxicological properties such as beauvericin (BEA), enniatins (ENNs), and moniliformin (MON), a group of lesser-studied toxins called emerging mycotoxins [11] (a non-exhaustive list of mycotoxin producing *Aspergillus, Penicillium* and *Fusarium* species, split into eight groups, is provided in Table 1). Even if these mycotoxins are found all over the world in foodstuffs and feedstuffs due to trade in these commodities that contributes to their worldwide dispersal. Moreover, Table 1 shows that one mycotoxin can be produced by several fungi, and that a fungus can produce several mycotoxins.

Several authors have shown an interest in cellular mechanisms and cellular toxicity in response to mycotoxin exposure. After ingestion by the consumer, the intestinal epithelium is the first host defense barrier against mycotoxins. However, although these cells are the first to be exposed to mycotoxins and at higher doses than other tissue cells, studies on the effect of mycotoxin mixtures on the gastrointestinal tract are scarce. Grenier and Appelgate [12] summarized in a recent review findings following major mycotoxin exposure (AFs, OTA, DON, T2, ZEA, and FUM) on digestive and absorptive functions, intestinal defense and microbiome composition. Briefly, they highlighted the large variability of mycotoxin bioavailability according to the considered mycotoxins and animal species. For example, the authors reported that more than 80% of AFs are absorbed within the gastrointestinal tract regardless of the non-ruminant species (via passive transport), whereas absorption of other major mycotoxins (TCT, OTA, or FUM) may vary from 1% to 60% (via passive transport by simple diffusion for OTA or *via* the paracellular route for DON). Moreover, several mycotoxins have been shown to undergo entero-hepatic circulation. This makes the mycotoxins available again *via* the bile in the entero-hepatic cycle, resulting in reabsorption and a prolonged retention time in the gastrointestinal tract. Intestinal metabolism in the gut epithelium and by the gut microbial population, limits the toxic effects of mycotoxins within the gastrointestinal tract. In particular, due to rumen microorganisms, ruminants are able to convert many mycotoxins into non-toxic metabolites before absorption, whereas for monogastrics, mycotoxin intestinal biotransformation takes place predominantly in the large intestine and thus provides little detoxification prior to absorption. However, little is known about the intestinal absorption and bacterial metabolism of the metabolites. Nevertheless, a recent in vitro study showed that the derivative 15-ADON caused the highest paracellular permeability and chemokine secretion compared to DON and 3-ADON in human intestinal cells Caco-2 [13]. Even if commensal microbiota is a key player in the detoxification against mycotoxins and their derivatives, it is important to note the potential of mycotoxins to enhance the toxic effects of intestinal pathogens and to change the intestinal microbiota balance by increasing the number of aerobic bacteria and thereby acting as a potential risk factor for chronic inflammatory diseases [12].

Because of their occurrence and toxicity, major mycotoxins (*i.e.*, AFs, OTA, ZEA, FUM, and DON) are the focus of legal regulations or guidance in many countries. The Joint Expert Committee on

Food Additives (JECFA), a scientific advisory body of the World Health Organization (WHO) and the FAO, evaluates mycotoxin risks. In the United States and the European Union, regulatory and recommended guidance for mycotoxins are issued by the FDA and the European Commission (EC) advised by the European Food Safety Authority (EFSA), respectively. To protect animal and human consumers, these regulations fixed regulatory threshold values in food and feed to ensure they are not harmful and recommended good agricultural practice. For example, the maximum levels (MLs) of EU regulatory limits range from 0.1 μ g/kg for aflatoxin B1 (AFB1) in processed cereal-based foods for human infants and young children, to 4000 μ g/kg for fumonisins B1 and B2 in unprocessed maize for human consumption. Concerning milk and milk-based products, MLs are $0.05 \,\mu g/kg$ for aflatoxin M1 (European Commission (EC) 2006 and subsequent amendments) [14]. Mycotoxin regulations differ across states, even if harmonization efforts are being undertaken in some trade zones. However, this harmonization would not necessarily be beneficial from a human health protection point of view because of the differences in contamination levels and dietary habits in various parts of the world [15]. In the developed world, the dietary exposure is below the mycotoxin tolerance limits and tolerable daily intakes established by the JECFA, but it is not always the case for developing countries, as reported by Shepard [16], with the example of maize-based diet. Moreover, with the intensive farming due to an increasing world population, and particularly in developing countries, the number of world inhabitants over-exposed to mycotoxins could be enhanced over the next few years.

Noteworthy, mycotoxins may occur in modified forms from their parent compounds, due mainly to plant detoxification systems. Indeed, as part of their defense against xenobiotics, plants can alter the chemical structure of mycotoxins by modifications generated by enzymes involved in detoxification processes. Because these modifications lead to modified chromatographic profiles, epitope conformation or polarity, these mycotoxin derivatives usually escape conventional analytical methods and are not regulated by legislation and thus are called "masked" mycotoxins. Even if in the case of several studied conjugated mycotoxins, a lower toxicity was observed compared to the parent compounds, a potential increased bioavailability during digestion of masked mycotoxins still represent a health threat [17,18]. As masked mycotoxins are an emerging issue and insufficient toxicological and quantification data are available, these metabolites will not be considered in this review.

Concerning the routinely screened mycotoxins, the current regulations were established on toxicological data from studies taking into account only one mycotoxin exposure at a time, and do not consider the combined effects of mycotoxins. However, the natural co-occurrence of mycotoxins in cereals grains is well-known, and can be explained by at least three reasons: (i) most fungi are able to produce several mycotoxins concurrently (Table 1); (ii) food commodities can be contaminated by several fungi simultaneously or in quick succession and (iii) animal diets are usually made up of multiple grain sources. This is supported by a three-year worldwide survey that indicates that 48% of 7049 analyzed feedstuffs samples were contaminated by two or more mycotoxins [19]. This multi-contamination risk exposure is particularly true for ruminants, which have varied diets compared to other farm animals. In particular, ruminants are fed with forages, which are commonly contaminated with several mycotoxins, as reported in the recent review from Gallo *et al.* [20]. These authors highlighted the lack of data concerning mycotoxin occurrence in silages and other forage crops, and recommended to analyze forages for nutritive and fermentative characteristics, but also mainly for mycotoxin contaminations.

The toxicity of mycotoxins combinations cannot always be predicted based upon their individual toxicities. Multi-exposure may lead to additive, synergistic or antagonistic toxic effects [6,21]. The data on combined toxic effects of mycotoxins are limited, thus the health risk from this multi-exposure is not well-known.

The aims of this present review are to display the main natural mycotoxin mixtures found in common foods, such as cereals, nuts, fruits, milk and processed products thereof, and feedstuffs, to summarize current regulations as well as the published experiments on these mycotoxin mixtures, and

to describe their known toxicological effects. This work may potentially underline areas lacking data for better taking into consideration this problem.

2. Mycotoxin Regulations

The last survey of the FAO in 2003 reported that, on a worldwide basis, around 100 countries, representing approximatively 87% of the world population, had regulations or detailed guidelines for mycotoxins or groups of mycotoxins in food and/or feed. Because of the various factors playing a role in the decision-making process to establish mycotoxin limits, including scientific, economic and political factors, the permitted limits and the mycotoxins targeted by legislation vary from country to country [15]. For example, the European Commission (EC) has issued maximum permitted levels for six groups of mycotoxins for animal feed: AFs, OTA, ZEA, FUM, DON, and rye ergot, and seven groups for human food: AFs, OTA, ZEA, FUM, DON, patulin (PAT) and citrinin (CIT); whereas only three groups are regulated by the FDA for animal feed (AFs, FUM, and DON) and one more for human food (PAT).

2.1. Aflatoxins

Regarding total aflatoxins (*i.e.*, sum of AFB1, AFB2, AFG1, and AFG2) in human food, EU MLs are 4 μ g/kg for peanuts and other oilseeds, tree nuts, dried fruits, cereals, and processed products thereof, intended for direct human consumption or use as ingredient in foodstuffs; 10 μ g/kg for tree nuts, dried fruits, maize and rice subjected to sorting, or other physical treatment, before human consumption as well as spices, dried figs, almonds, pistachios, apricot kernels, hazelnuts, and Brazil nuts intended for direct human consumption; and 15 μ g/kg for peanuts and other oilseeds, almonds, pistachios, apricot kernels, hazelnuts, and Brazil nuts intended for direct human consumption; and 15 μ g/kg for peanuts and other oilseeds, almonds, pistachios, apricot kernels, hazelnuts, and Brazil nuts subjected to sorting, or other physical treatment, before human consumption [14]. The FDA action level is 20 μ g/kg for total AFs in peanuts, Brazil nuts, pistachios, and other foods for direct human consumption [22].

Regarding animal feed, EU MLs have been issued for aflatoxin B1 only and range from 20 μ g/kg for feed materials to 10 μ g/kg for complementary and complete feed, with the exception of compound feed for young animals (MLs are 5 μ g/kg) [23]. In comparison, the action levels established by the FDA for AFs range from 20 μ g/kg for corn, peanut products, and other animal feeds and ingredients for immature and dairy animals, to 100 μ g/kg for corn and peanut products for breeding cattle, breeding swine and immature poultry, 200 μ g/kg for finishing swine and 300 μ g/kg for finishing beef cattle as well as cottonseed meal for beef, cattle, swine or poultry, regardless of age or breeding status [22].

For milk and milk-based products, only aflatoxin M1 is considered, and EU MLs are $0.05 \ \mu g/kg$ [14]. Indeed, AFM1 is metabolized and excreted in the milk after the ingestion of its parent molecule, AFB1, by dairy cattle. The action levels established by the FDA are 10 times higher than the EU MLs for AFM1 in milk (namely $0.5 \ \mu g/kg$) [22].

2.2. Ochratoxin A

OTA MLs in the EU are 0.5 μ g/kg for processed cereal-based foods and baby foods; 2 μ g/kg for wine, grape juice, grape nectar and grape must intended for direct human consumption; 3 μ g/kg for products derived from unprocessed cereals; 5 μ g/kg for unprocessed cereal, roasted coffee beans and ground roasted coffee; 10 μ g/kg for dried vine fruit and soluble coffee; 15 μ g/kg for certain spices; 20 μ g/kg for liquorice root for herbal infusion and 80 μ g/kg for liquorice extract for use in food in particular beverages and confectionery [14].

Mycotoxin	Fungi Source	Product of Primary Concern	Geographical Occurrence
AFs * (B1, B2, G1, G2)	Aspergillus (bombycis, flavus, nomius, ochraceoroseus, parasiticus, parvisclerotigenus, pseudotamarii, rambellii, toxicarius)	Cereals and cereal-based products (mainly corn), nuts, nut products and seeds, dried fruits, spices, milk and dairy products, meat, eggs	Temperate, tropical and subtropical regions (Southern Asia and Africa)
OTA *	Aspergillus (alliaceus, auricomus, carbonarius, cretensis, flocculosus, glaucus, lacticoffeatus, meleus, niger, ochraceus, pseudoelegans, roseoglobulosum, sclerotioniger, sclerotiorum, steynii, sulphureus, westerdijkiae); Penicillium (nordicum, verrucosum)	Cereals and cereal-based products (mainly rice and wheat), coffee and cocoa beans; wine, beer, dried fruits, spices, meat	From cool-temperate to tropical regions (Northern and Southern America, Northern and Western Europe, Africa and South Asia)
TCTs * (DON, NIV, T-2, HT-2, DAS)	Fusarium (acuminatum, armeniacum, culmorum, crookwellense, equisetii, graminearum, kyushuense, langsethiae, poae, pseudograminearum, sambucinum, scirpi, sporotrichioides, venamtum)	All cereals and cereal-based products	Northern temperate regions (Europe, America and Asia)
ZEA *	Fusarium (crookwellense, culmorum, equiseti, graminearum, incarnatum, pseudograminearum, semitectum, sporotrichioides, verticillioides)	All cereals and cereal-based products, and banana	Northern temperate regions (Europe, America and Asia)
FUM * (B1, B2, B3)	Fusarium (anthophilum, dlamini, fujikuroi, globosum, napiforme, nygamai, oxysporum, polyphialidicum, proliferatum, pseudonygamai, thapsinum, verticillioides)	Corn, millet, sorghum, rice and their derivatives	Hot-temperate regions (Europe, Africa)
BEA *	Fusarium (acuminatum, armeniacum, anthophilum, avenaceum, beomiforme, dlamini, equiseti, fujikuroi, globosum, langsethiae, longipes, nygamai, oxysporum, poae, proliferatum, pseudoanthophilum, sambucinum, semitectum, sporotrichioides, subglutinans)	All cereals and cereal-based products	Temperate regions (Europe)
ENs * (A, A1, B, B1)	Fusarium.(acuminatum, avenaceum, langsethiae, lateritium, poae, proliferatum, sambucinum, sporotrichioides, tricinctum)	All cereals and cereal-based products	Temperate regions (Europe)
MON *	Fusarium (acuminatum, avenaceum, culmorum, equiseti, fujikuroi, napiforme, nygamai, oxysporum, proliferatum, pseudonygamai, sporotrichioides, subglutinans, thapsinum, tricinctum, verticillioides)	All cereals and cereal-based products	Temperate regions (Europe)

Table 1. Some mycotoxins of interest and their fungal source, with primary food and feed hosts and endemic regions. References: [1,2,4,24–32].

* Abbreviations: aflatoxins (AFs); ochratoxin A (OTA); trichothecenes (TCTs); deoxynivalenol (DON); nivalenol (NIV); T-2 toxin (T-2); HT-2 toxin (HT2); diacetoxyscirpenol (DAS); zearalenone (ZEA); fumonisins (FUM); beauvericin (BEA); enniatins (ENs); moniliformin (MON).

For animal consumption, MLs are 250 μ g/kg for feed materials, 50 μ g/kg for complementary and complete feeding stuffs for pigs, and 100 μ g/kg for poultry [33].

The FDA does not establish regulatory guidance for this toxin.

2.3. Fumonisins

Concerning FUM, the EC has set MLs for the sum of fumonisins B1 and B2, ranging from 200 μ g/kg for processed cereal-based and baby foods for infants and young children, to 4000 μ g/kg for unprocessed maize. FUM may also be found in other common foods such as maize and maize-based foods intended for direct human consumption (MLs are 1000 μ g/kg), or maize-based breakfast cereals and snacks (MLs are 800 μ g/kg) [14]. The FDA guidance levels for the sum of fumonisins B1, B2, and B3 are between 2000 μ g/kg and 4000 μ g/kg for maize and maize-based products intended for human food [34].

Moreover, MLs for the sum of FB1 and FB2 are 60,000 μ g/kg for maize and maize products in feed materials and range from 5000 μ g/kg to 50,000 μ g/kg for complementary and complete feeding stuffs, depending on the species and the age of the animal (MLs are 5000 μ g/kg for pigs, equids, rabbits and pet animals, 10,000 μ g/kg for poultry, calves, lambs and kids, and 50,000 μ g/kg for adult ruminants and mink) [33]. The FDA guidance levels for the sum of FB1, FB2, and FB3 range from 5000 μ g/kg to 100,000 μ g/kg for corn and corn by-products in animal feed according to species and age (FDA guidance levels are 5000 μ g/kg for equids and rabbits, 20,000 μ g/kg for swine and catfish, 30,000 μ g/kg for breeding ruminants, poultry and mink, 60,000 μ g/kg for ruminants being raised for slaughter and mink being raised for pelt production, 100,000 μ g/kg for poultry being raised for slaughter, and 10,000 μ g/kg for all other species and classes of livestock) [34].

2.4. Zearalenone

EU MLs for ZEA in human food are 20 μ g/kg for processed maize-based foods for infants and young children, and processed cereal-based foods; 50 μ g/kg for bread, pastries, biscuits, cereal snacks and breakfast cereals; 75 μ g/kg for cereals intended for direct human consumption; 100 μ g/kg for maize, maize-based snacks, maize-based breakfast cereals and unprocessed cereals; 350 μ g/kg unprocessed maize and 400 μ g/kg for refined maize oil [14]. The FDA does not establish regulatory guidance for this toxin.

For feed materials, MLs range from 2000 μ g/kg for cereals and cereal products, to 3000 μ g/kg for maize products. Concerning complementary and complete feeding stuffs, MLs range from 100 μ g/kg for piglets and young sows, to 250 μ g/kg for sows and fattening pigs and 500 μ g/kg for calves, dairy cattle, sheep, and goats [33].

2.5. Trichothecenes

Regarding DON in human food, MLs range from 200 μ g/kg for processed cereal-based and baby foods to 1750 and 1250 μ g/kg for unprocessed durum wheat, oats, and maize as well as other unprocessed cereals, respectively. DON may also be found in other common foods such as cereals intended for direct human consumption and pasta (in this case MLs are 750 μ g/kg), as well as bread, pastries, biscuits, cereal snacks, and breakfast cereals (MLs are 500 μ g/kg) [14]. The FDA advisory level for DON is 1000 μ g/kg for finished wheat products intended for direct human consumption [35], and thus is close to the EU MLs. Currently, levels are under discussion for the sum of T-2 and HT-2 toxins in unprocessed cereals and cereals products for human consumption in the EU [14].

For feed materials, the EU MLs range from 8000 μ g/kg for cereals and cereal products, to 12,000 μ g/kg for maize by-products. EU MLs for complementary and complete feeding stuffs are 5000 μ g/kg expect for pigs (MLs are 900 μ g/kg) and calves, lambs and kids (MLs are 2000 μ g/kg) [33]. For grain and grain by-products in animal feed, the FDA advisory levels range from 5000 μ g/kg to 10,000 μ g/kg (according to the considered species and the age of the animal) [35], whereas due to the relatively low human exposure to the other TCTs, such as nivalenol (NIV) and diacetoxyscirpenol

(DAS), and their co-occurrence with typically more abundant DON, establishing maximum permitted levels for these toxins is currently not considered [14]. However, due to their possible additive or synergistic toxic effects, it would be interesting to establish regulations for total TCTs, as it is already the case with AFs and FUM.

2.6. Other Regulated Mycotoxins

Regarding patulin (PAT), the EU MLs are 10 μ g/kg for apple juice and solid apple products, including apple compote and apple purée, for infants and young children. Moreover, MLs are 25 μ g/kg for solid apple products for direct human consumption and 50 μ g/kg for fruit juices, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice [14]. FDA regulatory limits are 50 μ g/kg for apple juice and apple juice component of a food that contains apple juice as an ingredient [36].

EU MLs for citrinin (CIT) are 2000 μ g/kg for food supplements based on rice fermented by the "red yeast" *Monascus purpureus* [14].

MLs for rye ergot in the EU are 1000 mg/kg for feed materials and compound feed containing ungrounded cereals [33].

Thus, the European Community has one of the most stringent regulations in the world, with numerous mycotoxins and commodities concerned, and more restrictive levels. However, like the other regulations in the world, the EC does not consider the combined toxicological effects of mycotoxins.

3. Natural Co-Occurrence of Mycotoxins in Foods and Feeds

Several surveys reported the natural co-occurrence of mycotoxins from all over the world, and most of them concerned the major mycotoxins AFs, OTA, ZEA, FUM, and TCTs—especially DON. However, only a few studies specified the number of co-occurring mycotoxins with the percentage of the co-contaminated samples, as well as the main combinations found. We selected the relevant data and papers (from 1987 to present) from over a hundred papers dealing with mycotoxin co-occurrence in different foods and feeds. Only studies with at least 10 samples were considered.

As presented in Figure 1a, more than 60% of the information comes from Europe, whereas merely 7% is obtained from North America, and only one paper studied samples from Oceania. Concerning the commodity types, raw and processed cereals are the most frequently studied, representing 80% of the overall data. The rest of the data mainly concerns plant products, especially fruits, spices, and nuts, and only a few studies were focused on milk and its derivatives (Figure 1b). Overall, about 50% of the data concerning cereals and cereal based-products comes from Europe (data not shown). Additionally, amongst the 107 included studies [37–143], about 35% was published between 2011 and 2015, highlighting the increasing interest for worldwide mycotoxin co-occurrence.



Figure 1. Data distribution depending on (**a**) geographic regions and (**b**) commodities. Data compiled from 107 articles. References: [37–143].
The difficulty of comparing studies using different methodologies of mycotoxin detection and quantification should be emphasized, considering their associated sensitivity and accuracy variations. Indeed, since 1972, we have witnessed a tremendous evolution of chromatographic and immuno-techniques. Especially since 2011 with the development of LC or GC-MS/MS that can detect ever more co-occurring mycotoxins. Moreover, some authors focused on only certain mycotoxins while others developed non-targeted approaches, which also complicates qualitative and quantitative comparisons. For example, the last worldwide mycotoxin survey [144] found up to 75 co-occurring mycotoxins in a same sample from a LC-MS/MS analysis targeting more than 380 mycotoxins simultaneously, whereas up to seven co-occurring mycotoxins were found in a same sample among the 107 papers analyzed, with a more "classic" approach targeting less than 15 major mycotoxins [123].

The main mixtures reported in these articles were analyzed by commodity type (cereals and cereals based-products, herbs and spices, dried fruits, fruits and vegetables, oilseeds, and milk and its derivatives) and by region (Europe, Africa, Asia, South America, and North America). Because only one study cites a sample coming from New Zealand, Oceania was not included.

3.1. Results by Commodity Type

Among the 116 mycotoxin combinations found by the authors in cereal and derived cereal product samples, AFs+FUM, DON+ZEA, AFs+OTA, and FUM+ZEA were the most present. These mixtures are quoted 21, 14, 12, and 11 times out of the 91 papers analyzing cereal products, respectively, representing 23%, 15%, 13%, and 12% of these articles respectively. Furthermore, the last survey by the BIOMIN Company showed that DON, FUM, and ZEA are the most prevalent mycotoxins in the world, with a prevalence of 66%, 56%, and 53%, respectively, among the 6844 analyzed agricultural commodity samples [144]. Because of their common co-occurrence, also potentially associated with AFs (with a worldwide prevalence of 22%) [144], these mycotoxin toxicological interactions must not be disregarded.

Only four papers focused on herbs and spices [68,75,110,122]. In all of them, AFs+OTA mixtures were listed. The other combinations found corresponded to OTA+ZEA, AFs+ZEA, and AFs+OTA+ZEA, quoted twice for OTA+ZEA and AFs+OTA+ZEA, and once for AFs+ZEA.

Dried fruits were also studied in four papers [45,68,74,127]. In this context, the AF<u>s+</u>OTA mixture was cited three times and AFs + cyclopiazonic acid (CPA) only once.

Among the three articles concerning fruits and vegetables, apples have been extensively studied [73,101,124]. Five mycotoxin mixtures were reported in these articles and none of the authors found the same mixtures. It should be noted that PAT was quoted twice in combination with either AFs or CIT.

The same observation was made for oilseeds (nuts, tree nuts, soy, olives): among the 11 mixtures quoted in six papers, all are cited only once [59,60,63,120,122,123]. The combinations listed were mainly formed with TCTs.

Concerning milk and its derivatives, mainly cheeses, only three mixtures have been reported: Roquefortine-C (ROQ-C) + mycophenolic acid (MYC-A), AFs+OTA, and AFs+CPA. These combinations were quoted 2-, 2- and 1-times out of five articles, respectively [42,66,86,91,108]. Other animal products, like meat or eggs, have not been studied in a co-occurrence context.

Using this literature set, it can be summarized that AFs are found in various food and feed products, often in combination with OTA or fusariotoxins (mainly FUM and ZEA). Generally, binary mixtures are the most common among about 25 mycotoxins studied in the 107 papers, even if the last BIOMIN survey showed, that among the worldwide samples tested on average 30 different metabolites were detected per sample using a multi-mycotoxin technique (LC-MS-MS) [144].

The relation between geographical origin and reported mycotoxin combinations is presented in Figure 2. For European samples, among the 105 mycotoxin mixtures found, the most reported one (16 out of 67 publications, or 24%) was AFs+OTA. While, DON+ZEA, DON+NIV, and DON+T2 combinations were quoted in 15%, 13%, and 12% of these articles, respectively. The other combinations were listed in less than 10% of the articles.



Figure 2. Main mycotoxin mixtures quoted in the papers depending on their geographic origin. Data compiled from 107 articles. References: [37–143].

Concerning African samples, over the 26 observed mycotoxins combinations, AFs+OTA was once again, the main mixture, representing 35% of the 14 publications related to African samples. The AFs+FUM and AFs+ZEA binary combinations as well as the AFs+OTA+ZEA ternary combination were cited in 29%, 21%, and 29% of these articles, respectively. The other mixtures were observed in only two or less articles.

In Asia, AFs+FUM was the most observed mixture (seven out of nine articles, or 78%) among the 18 listed combinations. The other combinations were reported in only one or two articles. It can be highlighted that AFs or FUM were present in almost all the other mixtures.

In South America, more particularly in Brazil and Argentina, AFs+FUM was also the most observed mixture, as it was reported in 50% (six out of 12 articles). While FUM+ZEA was the second most observed combination (25%) among the 12 listed mycotoxins mixtures.

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Concerning the seven publications from North America, 21 mycotoxin combinations were reported, the main ones being DON+ZEA and DON+DAS+T2, quoted in two papers (29%), respectively.

In conclusion regarding the occurrence and prevalence aspect, the AFs+FUM mixture is the most prevalent in Africa, Asia, and South America (Figure 2). Maize harvested in the tropical and subtropical areas of the world with hot and humid climates is the major commodity contaminated with the two toxins. Aflatoxins are a far greater problem in the tropics than in temperate zones of the world. However, because of the movement of agricultural commodities around the globe, no region of the world is aflatoxin-free. In more temperate and cold regions (Europe and North America), mixture of TCTs or TCTs with ZEA are the most common, highlighting the importance of the climate conditions on fungal contamination, growth, metabolism and thus mycotoxin mixtures. *Fusarium* is the main genus implicated in TCTs production and many toxigenic *Fusarium* species have been associated with infected grain. The geographical distribution of the *Fusarium* species is probably related to environmental temperature requirements and/or different agricultural practices [145].

Overall, among the 127 mycotoxin mixtures described by the authors from all combined countries and commodities, the main mycotoxin mixtures cited were AFs+OTA, AFs+FUM, and DON+ZEA, found in 21%, 20%, and 13% of the studies. Cereals represent the main OTA and ZEA sources of human intake [146,147]. Among cereal grains, AFs and ZEA mainly appear in corn (EFSA, 2004; EFSA 2007), whereas barley has a particularly high likelihood of OTA contamination [148]. Over the past few years, there has been emerging evidence of potential aflatoxin contamination of feed materials grown in areas of southern Europe, where a subtropical climate and extensive agricultural practice favor fungal growth and the subsequent formation of aflatoxins (EFSA, 2007). However, it is important to note that our analysis did not consider the "year" parameter, and it is well known today that prevalence and contamination levels of mycotoxins vary greatly according to harvest year of the cereals [149]. Moreover, climatic and agricultural practice changes observed over the last years, including the reduction of fungicide use, could lead to mycotoxin contamination in food [150,151].

Based on the data organized by region, a dendogram was created using the "HeatMap" function of the "R Project for Statistical Computing" software and a hierarchical ascendant classification analysis using the "hclust" function and with the default parameter "ward's method". This graphic representation, corresponding to a qualitative approach, is a heat-grey plot matrix illustration, in which the grey color intensity depends on the number of times that a mycotoxin combination mixture is cited (Figure 3). Asia and South America exhibit similar profiles; they are as close to Africa's profile as the same mixtures, with a similar number of reports, have been observed. Despite the fact that EU regulations are one of the most stringent in the world, Europe exhibits a large range of mixtures cited compared to the other regions but it is worth nothing that European studies were more extensive as they represent 61% of the 106 studied articles. Thus, the significant difference in the number of publications by region could also impact on the results. Nevertheless, North America has the closest profile to Europe. This analysis was supported by the above comments which highlighted the role of climate in mixture occurrence and potentially by similar agricultural methods.



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Figure 3. All mycotoxin mixtures quoted in the papers depending on their geographic origin. (\Box = mixtures no cited; = mixtures cited between 1 and 3 times; = mixtures cited between 3 and 5 times; = mixtures cited between 5 and 7 times; = mixtures cited between 7 and 9 times; = mixtures cited more than 9 times). Reading from left to right on the *x*-axis:

1. AFs FUM 2. DON ZEA 3. AFs OTA 4. FUM ZEA 5. DON NIV 6. DON T2 7. DON HT2 8. AFs ZEA 9. FUM DON 10. FUM OTA 11. DON T2 ZEA 12. T2 HT2 13. BEA ENNs 14. AFs OTA ZEA 15. DON ADON NIV 16. DON ADON ZEA 17. AFs FUM ZEA 18. FUM DON ZEA 19. DON ADON 20. DON OTA 21. FUM NIV 22. OTA ZEA 23. OTA CIT 24. DON T2 HT2 25. DON HT2 NIV 26. DON HT2 ZEA 27. DON NIV ZEA 28. FUM ZEA OTA 29. DON ADON HT2 NIV 30. DON T2 HT2 ZEA 31. DON FUS-X 32. ADON ZEA 33. AFs NIV 34. DON ADON HT2 35. DAS T2 HT2 36. AFs FUM DON 37. DON ADON NIV ZEA 38. AFs FUM OTA ZEA 39. DON ADON T2 HT2 NIV 40. DAS T2

41. DAS HT2 42. T2 NIV 43. T2 ZEA 44. NIV BEA 45. ENNs FUS 46. AFs DON 47. FUM BEA 48. FUM MON 49. OTA NIV 50. DON ADON T2 51. DON DAS HT2 52. DON T2 NIV 53. DON HT2 FUS-X 54. DON OTA ZEA 55. AFs FUM NIV 56. AFs BEA NIV 57. DON ADON T2 NIV 58. DON ADON T2 ZEA 59. DON T2 HT2 NIV 60. DON T2 NIV ZEA 61. DON ADON T2 HT2 ZEA 62. DON DAS 63. DON aZOL 64. DON ENNs 65. NIV HT2 66. NIV FUS-X 67. NIV ZEA 68. ZEA ENNs 69. BEA FUS 70. FUM T2 71. FUM FUS 72. MYC-A ROQ-C 73. DON DAS T2 74. DON NIV FUS-X 75. DON NIV MAS 76. DON ZEA α ZOL 77. T2 T2tetraol HT2 78. T2 HT2 ZEA 79. NIV FUS-X BEA

80. BEA ENNs FUS

81. BEA ENNs MON 82. AFs OTA DON 83. AFs OTA T2 84. AFs OTA NIV 85. AFs OTA FUM 86. AFs FUM T2 87. AFs DON ZEA 88. FUM DON NIV 89. FUM T2 HT2 90. FUM BEA OTA 91. FUM BEA FUS 92. FUM OTA CIT 93. DON ADON T2 HT2 94. DON ADON HT2 ZEA 95. DON ADON ZEA αZOL 96. DON MAS NIV ZEA 97. DON DAS T2 HT2 98. DON T2 ZEA α ZOL 99. DON HT2 NIV ZEA 100. DAS T2 HT2 ZEA 101. T2 HT2 MAS ZEA 102. T2 HT2 NIV BEA 103. NIV ZEA BEA ENNs 104. AFs OTA DON ZEA 105. AFs OTA T2 ZEA 106. FUM DON NIV ZEA 107. DON ADON MAS HT2 ZEA 108. DON ADON HT2 NIV ZEA 109. DON ADON HT2 NIV FUS-X 110. DON DAS T2 HT2 ZEA 111. DON MAS T2 HT2 ZEA 112. DON T2 HT2 NIV ZEA 113. MAS T2 HT2 NIV ZEA 114. AFs FUM OTA DON ZEA 115. DON ADON T2 NIV ZEA α ZOL 116. DON ADON NIV ZEA α ZOL β ZOL

4. Toxicological Impact of Mycotoxin Interactions

As stated previously, toxicological evaluation and therefore regulations are based so far on individual mycotoxin. However, as confirmed by the data analysis presented in the first part of this review, single mycotoxin contamination is not the norm but rather the exception. It is therefore of the utmost importance to evaluate the toxicological impact of mycotoxin combinations to better reflect feed and food contamination and their associated animal and human health risks. In this context, Grenier and Oswald [6] reviewed in vivo experiments until 2010, in which laboratory and farm animals were exposed to a combination of mycotoxins, and described the type of observed interactions. Since 2011, only few *in vivo* studies have been published. In the framework of this review, we focused on *in vitro* experiments published between 1980 and 2015. Indeed, even if cell cultures have many limitations such as immortalization, limited survival or metabolic imbalance, in vitro models are more and more used for understanding the mechanisms of mycotoxin action and their mixtures, especially toxicity on cell-specific function [152]. Among the 58 analyzed articles, 50% were published during the last five years showing the interest of this approach as an alternative of interest to animal models. In this context, in vitro studies become embedded in national and international legislation regulating the use of animals in scientific procedures in order to encourage and develop the principles of the 3Rs (Replacement, Reduction, and Refinement) as a framework for humane animal research.

Most of the selected publications concern the effect of binary mixtures. Indeed, among the 93 studied mycotoxin mixtures, 70% corresponded to binary mixtures, 24% to ternary mixtures, and 6% were quaternary or quinary mixtures. Furthermore, the main studied mixtures were OTA+CIT, DON+NIV, DON+T2, OTA+AFB1, and OTA+FB1 found in 28%, 14%, 12%, 10%, and 9% of the articles, respectively. Another observation corresponds to the fact that mixtures involving fusariotoxins were the most studied, representing about 70% of all the analyzed mixtures, with 50% involving exclusively fusariotoxins and 22% are formed with OTA.

Concerning cell models, 43% of the authors used, *inter alia*, cells from human origin, 26% porcine models, 19% murine models, and more marginally monkey, bovine, fish, turkey or/and even yeast, which is a simple model to examine the immediate effects of mycotoxins on growth inhibition or CO_2 production for example (Figure 4a). Overall, more than 30 different cell lines were used among the 58 articles studied, and most of these cells came from kidney, blood, intestine, and liver (Figure 4b). More particularly, Caco-2 (human epithelial colorectal adenocarcinoma cells), PK15 no copyright permission needed as we created this figure (porcine hepatocellular carcinoma cells), Vero (monkey renal proximal tubular epithelial cells), and HepG2 (human kidney epithelial cells) were the most used cell models as they were reported in 8, 8, 7, and 5 articles, respectively. This is linked to the fact that these cell models.





Regarding the studied parameters, cell viability was the main endpoint used by the authors (in 64% of the studies), followed by cell apoptosis or/and necrosis (19%), DNA damage (17%) and oxidative damage (16%). Some authors were also interested in macromolecule synthesis (RNA, DNA, proteins), or immunotoxicity parameters. Moreover, all these tests are performed between 0 and 72 h (acute exposure), except in the work of Fichetic *et al.* [166], in which mycotoxin interactions were studied during 14 days (chronic exposure). In particular, for cell viability, studies were mostly carried out on 24 h and/or 48 h, with the most commonly used being the tetrazolium reduction assays.

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In the present review, we decided to focus on the *in vitro* effects of fusariotoxin mixtures on cell viability using mammalian cell models (Table 2). Concerning mycotoxin mixtures involving OTA, a review about their *in vitro* and *in vivo* combined effects was recently published [211].

To better understand the conclusions presented by the authors about the *in vitro* effects of fusariotoxin mixtures, the main types of interactions between mycotoxins, as well as mathematical models for characterizing these interactions, are described hereafter.

4.1. Characterization of the Different Interactions Between Mycotoxins

Mycotoxin interactions can be classified in three main different categories: antagonistic, additive, and synergistic. Depending on the authors, more categories may be distinguished, namely potentiation and less-than-additive, often classified in synergistic and antagonistic effects, respectively. Figure 5 illustrates the possible different interactions of mycotoxins with the example of cell viability measure.

Additivity is mentioned when the effect of the combination could be calculated as the sum of the individual effects of the two studied toxins (Figure 5a). Thus, additivity is *a priori* an absence of interaction.

Synergism is observed when the effect of the mycotoxin combination is greater than expected in comparison to the sum of the individual effects of the two studied mycotoxins (Figure 5b). In the case when one or both of the mycotoxins does not induce effect whereas the combination induces a significant effect, one can speak of potentiation (Figure 5c). However, very few studies use this term to categorize the effect, and most of them use synergism.

Antagonism is cited when the effect of the mycotoxin combination is lower than expected from the sum of the individual effects of the two studied mycotoxins (Figure 5d). If the effect of the mycotoxin combination mainly reflected the effect of the most toxic mycotoxin, without additional effect of the other mycotoxin, the term "less-than-additive" may be used.

Table 2. In vit	tro interactions	between	fusariotoxins o	n cell viability.

Mycotoxin Couples/Cells	Doses (µM)	Exposure		Toxicological Effect	References
		In	teraction between TC	Т	
DON+15-ADON	DON: 0.25-4	48 h	Synergistic	at low inhibitory concentration levels $(IC_{10, 20, 30})$	[155]
Human epithelial colorectal adenocarcinoma cells: Caco-2	15-ADON: 0.25-4		Additive	at medium inhibit concentration levels (IC $_{\rm 40,50})$	
DON+15-ADON	DON: 0.2-15	24 h	Synergistic	from IC ₁₀ to IC ₈₀	[154]
(ileum + jejunum): IPEC-1	15-ADON: 0.2-15		, ,		
DON+3-ADON	DON: 0.25-4	48 h	Synergistic	at low and medium inhibitory concentration levels (IC $_{10,\ 20,\ 30,\ 40})$	[155]
Human epithelial colorectal adenocarcinoma cells: Caco-2	3-ADON: 0.42-6.67	40 11	Additive	at the 50% growth inhibition level (IC $_{50}$)	[100]
DON+3-ADON Intestinal porcine epithelial cells (ileum + jejunum): IPEC-1	DON: 0.2–15 3-ADON: 2–150	24 h	Antagonistic Additive Synergistic	at low inhibitory concentration levels (IC_{10} – IC_{30}) at medium inhibitory concentration levels (IC_{30} – IC_{60}) at high inhibitory concentration levels (IC_{60} – IC_{80})	[154]
15-ADON+3-ADON	15-ADON: 0.25-4	48 h	Synergistic	at low cytotoxicity levels (IC _{10, 20, 30})	[155]
Human epithelial colorectal adenocarcinoma cells: Caco-2	3-ADON: 0.42-6.67	10 11	Additive	at medium inhibitory concentration levels (IC $_{\rm 40,50})$	[100]
15-ADON+3-ADON	15-ADON: 0.2-15	24 h	Synergistic	at all cytotoxicity levels (IC_{10} – IC_{80})	[154]
(ileum + jejunum): IPEC-1	3-ADON: 2-150		, 0	, , , , , , , , , , , , , , , , , , ,	
DON+15-ADON+3-ADON	DON: 0.25-4	40.1	Synergistic	at low cytotoxicity levels (IC _{10, 20, 30}) at the 40% growth inhibiting level (IC)	[155]
adenocarcinoma cells: Caco-2	3-ADON: 0.42–6.67	48 n	Antagonistic	from the 50% growth inhibition level ((C_{40})	[155]
DON+NIV	DON: 10-100	24 h 48 h and 72 h	Additivo	at 50% growth inhibition level (IC ₅₀)	[194]
Murine monocyte macrophage cells: J774A.1	NIV: 10–100	24 II, 40 II and 72 II	Additive		[1/1]
DON+NIV	DON: 0.5-2	48 h	Antagonistic	at the lowest dose	[209]
Intestinal porcine epithelial cells (jejunum): IPEC-J2	NIV: 0.5–2		Synergistic	at the highest dose	
DON+NIV	DON: 0.25-4	48 h	Synergistic	at all cytotoxicity levels (from IC_{10} to IC_{50})	[155]
Human epithelial colorectal adenocarcinoma cells: Caco-2	NIV: 0.2–3.2	10 11	-)		[]
DON+NIV	DON: 0.2-15	24 h	Synergistic	at all cytotoxicity levels (from IC_{10} to IC_{80})	[154]
Intestinal porcine epithelial cells (ileum + jejunum): IPEC-1	NIV: 0.2–15	2 1 11	-)	,,	[]
DON+FX	DON: 0.25-4	48 h	Synergistic	at all cytotoxicity levels (from IC_{10} to IC_{50})	[155]
Human epithelial colorectal adenocarcinoma cells: Caco-2	FX: 7.5–120		- ,	y	[]

Mycotoxin Couples/Cells	Doses (µM)	Exposure		Toxicological Effect	References
DON+FX Intestinal porcine epithelial cells (ileum + jejunum): IPEC-1	DON: 0.2–15 FX: 0.12–9	24 h	Antagonistic	at all inhibitory concentration levels (IC $_{10}$ -IC $_{80}$)	[154]
NIV+FX Human epithelial colorectal adenocarcinoma cells: Caco-2	NIV: 0.2–3.2 FX: 7.5–120	48 h	Synergistic Additive	at low cytotoxicity levels (IC $_{10, 20}$) at medium cytotoxicity levels (IC $_{30, 40, 50}$)	[155]
NIV+FX Intestinal porcine epithelial cells (ileum + jejunum): IPEC-1	NIV: 0.2–15 FX: 0.16–12	24 h	Additive	at all cytotoxicity levels (IC $_{10}$ -IC $_{80}$)	[154]
DON+NIV+FX Human epithelial colorectal adenocarcinoma cells: Caco-2	DON: 0.25–4 NIV: 0.2–3.2 FX: 7.5–120	48 h	Antagonistic Additive	at low cytotoxicity levels (IC $_{10, 20}$) at medium cytotoxicity levels (IC $_{30, 40, 50}$)	[155]
DON+T2 Chinese hamster ovary cells: CHO-K1	DON: 0.25–4 T2: 0.006–0.1	24 h, 48 h and 72 h	Antagonistic		[197]
DON+T2 Monkey kidney epithelial cells: Vero	DON: 0.25–8 T2: 0.001–0.05	24 h, 48 h and 72 h	Antagonistic		[198]
DON+T2 Hematopoietic progenitors: CFU-GM	DON: 0.04-0.1 T2: 0.0005-0.0016	14 days	Additive		[166]
		Interaction be	ween TCT and other	fusariotoxins	
DON+BEA Chinese hamster ovary cells: CHO-K1	DON: 0.25–4 BEA: 0.78–12.5	24 h, 48 h and 72 h	Antagonistic		[197]
DON+BEA Monkey kidney epithelial cells: Vero	DON: 0.25–8 BEA: 0.78–25	24 h, 48 h and 72 h	Antagonistic		[198]
DON+BEA Hematopoietic progenitors: CFU-GM	DON: 0.04-0.1 BEA: 0.064-3.2	14 days	Synergistic		[166]
T2+BEA Chinese hamster ovary cells: CHO-K1	T2: 0.006–0.1 BEA: 0.78–12.5	24 h, 48 h and 72 h	Synergistic		[197]
T2+BEA Monkey kidney epithelial cells: Vero	T2: 0.001–0.05 BEA: 0.78–25	24 h, 48 h and 72 h	Antagonistic		[198]
DON+FB1 Human epithelial colorectal adenocarcinoma cells: Caco-2	DON: 4–20 FB1: 10	72 h	Additive		[187]
DON+FB1 Hematopoietic progenitors: CFU-GM	DON: 0.04–0.1 FB1: 0.5–2	14 days	Antagonistic		[166]

Mycotoxin Couples/Cells	Doses (µM)	Exposure		Toxicological Effect	References
DON+FB1	DON: 0.5-2	48 h	Antagonistic	at the lowest dose	[209]
Intestinal porcine epithelial cells (jejunum): IPEC-J2	FB1: 20–40	10 11	Synergistic	at the highest dose	[]
NIV+FB1	NIV: 0.5–2	48 h	Antagonistic	at the lowest dose	[209]
(jejunum): IPEC-J2	FB1: 20–40		Synergistic	at the highest dose	
DON+ZEA	DON: 10-20	72 h	Additive		[187]
Human epithelial colorectal adenocarcinoma cells: Caco-2	ZEA: 10-20	,211	riddiive		[]
DON+ZEA	DON: 0.04-0.1	14 days	Additive		[166]
CFU-GM	ZEA: 0.2–10	, ,			
DON+ZEA	DON: 0.5-2	48 h	Antagonistic	at the lowest dose	[209]
Intestinal porcine epithelial cells (jejunum): IPEC-J2	ZEA: 10-40	10 11	Synergistic	at the highest dose	[==>]
DON+ZEA	DON: 100	24 h	Antagonistic		[156]
Human colon carcinoma cells: HCT116	ZEA: 40		0		J
NIV+ZEA	NIV: 0.5–2	48 h	Antagonistic	at the lowest dose	[209]
Intestinal porcine epithelial cells (jejunum): IPEC-J2	ZEA: 10-40		Synergistic	at the highest dose	[]
T2+ZEA	T2: 0.0005-0.0016	14 days	Additive		[166]
Hematopoietic progenitors: CFU-GM	ZEA: 0.2–10		- Manure		[]
T2+ZEA	T2: 0.025-0.1	24 h	Additive		[158]
Monkey kidney epithelial cells: Vero	ZEA: 0.025-0.1		- Manure		r 1
DON+T2+BEA	DON: 0.25-4				
Chinese hamster ovary cells: CHO-K1	T2: 0.006–0.1 BEA: 0.78–12.5	24 h, 48 h, and 72 h	Synergistic		[197]
DON+T2+BEA	DON: 0.25-8	24 h 49 h and 72 l	Antagonistic		[100]
Vionkey kidney epithelial cells: Vero	BEA: 0.78–25	24 n, 48 n, and 72 h	Amagonistic		[198]
DON+NIV+ZEA	DON: 0.5-2	10.1	Antagonistic	at the lowest dose	[200]
(jejunum): IPEC-J2	ZEA: 10–40	48 h	Synergistic	[209]	

Mycotoxin Couples/Cells	Doses (µM)	Exposure		Toxicological Effect	References
DON+NIV+FB1	DON: 0.5–2 NIV: 0.5–2	48 h	Antagonistic	at the lowest dose	[209]
Intestinal porcine epithelial cells (jejunum): IPEC-J2	FB1: 20-40		Synergistic	at the highest dose	
DON+ZEA+FB1 Human epithelial colorectal adenocarcinoma cells: Caco-2	DON: 10–20 ZEA: 10–20 FB1: 10	72 h	Additive		[187]
DON+ZEA+FB1 Intestinal porcine epithelial cells (jejunum): IPEC-J2	DON: 0.5–2Z EA: 10–40 FB1: 20–40	48 h	Antagonistic Synergistic	at the lowest dose at the highest dose	[209]
NIV+ZEA+FB1 Intestinal porcine epithelial cells (jejunum): IPEC-J2	NIV: 0.5–2 ZEA: 10–40 FB1: 20–40	48 h	Antagonistic Synergistic	at the lowest dose at the highest dose	[209]
DON+NIV+ZEA+FB1 Intestinal porcine epithelial cells (jejunum): IPEC-J2	DON: 0.5–2 NIV: 0.5–2 ZEA: 10–40 FB1: 20–40	48 h	Antagonistic Synergistic	at the lowest dose at the highest dose	[209]
		Interactio	on between other fusa	riotoxins	
ZEA+α-ZOL		24 h and 72 h	Antagonistic	at all cytotoxicity levels (from IC_{10} to IC_{90})	
Human hepatocellular carcinoma cells: HepG2	ZEA: $0.5-50$ α -ZOL: 1–100	48 h	Antagonistic Additive Synergistic	at IC _{10, 20, 30, 40} at IC _{50, 60, 70} at IC _{80, 90}	[208]
ZEA + α - ZOL Chinese hamster ovary cells:	ZEA: 12.5–50 α-ZOL: 6 25–25	24 h	Synergistic Additive	at low cytotoxicity level (IC ₂₅) at medium and high cytotoxicity levels (from IC ₅₀ to IC ₉₀)	[204]
CHO-K1	u 201.0.20 20	48 h and 72 h	Additive	at all cytotoxicity levels (from IC ₂₅ to IC ₉₀)	
ZEA+β-ZOL Chinese hamster ovary cells: CHO-K1	ZEA: 12.5–50 β-ZOL: 6.25–25	24 h, 48 h and 72 h	Additive	at all cytotoxicity levels (from IC_{25} to IC_{90})	[204]
	α-ZOL: 6 25-25	24 h	Additive	at all cytotoxicity levels (IC _{25, 50, 75, 90})	
α -ZOL+ β -ZOL Chinese hamster ovary cells:		48 h	Antagonistic	at low and medium cytotoxicity levels ($IC_{25, 50}$)	
СНО-К1			Additive	at high cytotoxicity levels ($IC_{75, 90}$)	[204]
	β-ZOL: 6.25–25 —	72 h	Additive Antagonistic	at low and high cytotoxicity levels (IC $_{25,75,90}$) at medium cytotoxicity level (IC $_{50}$)	
ZEA+α-ZOL+β-ZOL	ZEA: 12.5–5	24 h and 48 h	Antagonistic Synergistic	at low and medium cytotoxicity levels (IC $_{25, 50}$) at high cytotoxicity levels (IC $_{75, 90}$)	[204]

Mycotoxin Couples/Cells	Doses (µM)	Exposure		Toxicological Effect	References
Chinese hamster ovary cells: CHO-K1	α-ZOL: 6.25–25 β-ZOL: 6.25–25	72 h	Antagonistic Synergistic	at low cytotoxicity level (IC ₂₅) at medium and high cytotoxicity levels (IC _{50,75,90})	
ZEA+FB1 Human epithelial colorectal adenocarcinoma cells: Caco-2	ZEA: 5–20 FB1: 10	72 h	Antagonistic		[187]
ZEA+FB1 Intestinal porcine epithelial cells (jejunum): IPEC-J2	ZEA: 10–40 FB1: 20–40	48 h	Antagonistic Synergistic	at the lowest dose at the highest dose	[209]
ZEA+FB1 Human epithelial colorectal adenocarcinomia cells: Caco-2	ZEA: 10 FB1: 10	72 h	Antagonistic		[186]
BEA+FB1 Porcine renal proximal tubular epithelial cells: PK15	BEA: 0.064–6.4 μM FB1: 0.069–6.9 μM	24 h	Additive Antagonistic	at low doses at the highest dose	[179]
BEA+ENB Hematopoietic progenitors: CFU-GM	BEA: 0.064–3.2 ENB: 2–6	14 days	Additive		[166]
ENA+ENA ₁ Chinese hamster ovary cells: CHO-K1	ENA: 0.365–5 ENA ₁ : 0.625–5	24 h	Synergistic Additive	at low cytotoxicity levels (IC $_{25}$) at medium and high cytotoxicity levels (IC $_{50,75,90}$)	[189]
ENA+ENA₁ Human epithelial colorectal adenocarcinomia cells: Caco-2	ENA: 0.365–5 ENA ₁ : 0.625–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC ₅) at other fractions affected (IC _{25, 50, 75, 90})	[196]
ENA+ENB Chinese hamster ovary cells: CHO-K1	ENA: 0.365–5 ENB: 0.625–5	24 h	Synergistic Additive	at low and medium cytotoxicity levels (IC $_{25,50}$) at high cytotoxicity levels (IC $_{75,90}$)	[189]
ENA+ENB Human epithelial colorectal adenocarcinomia cells: Caco-2	ENA: 0.365–5 ENB: 0.625–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC ₅) at other fractions affected (IC _{25, 50, 75, 90})	[196]
ENA+ENB ₁ Chinese hamster ovary cells: CHO-K1	ENA: 0.365–5 ENB ₁ : 0.625–5	24 h	Additive	at all inhibitory concentration levels (IC $_{\rm 25,50,75,90})$	[189]
ENA+ENB ₁ Human epithelial colorectal adenocarcinomia cells: Caco-2	ENA: 0.365–5 ENB ₁ : 0.625–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC $_5$) at other fractions affected (IC $_{25}$, $_{50}$, $_{75}$, $_{90}$)	[196]
ENA ₁ +ENB Chinese hamster ovary cells: CHO-K1	EN A ₁ : 0.365–5 ENB: 0.625–5	24 h	Additive	at all inhibitory concentration levels (IC $_{\rm 25,50,75,90})$	[189]
ENA₁+ENB Human epithelial colorectal adenocarcinoma cells: Caco-2	EN A ₁ : 0.365–5 ENB: 0.625–5	24 h	Antagonistic Additive Synergistic	at the lowest fraction affected (IC ₅) at medium fractions affected (IC ₂₅ , $_{50}$, $_{75}$) at the highest fraction affected (IC ₉₀)	[196]

Mycotoxin Couples/Cells	Doses (µM)	Exposure		Toxicological Effect	References
ENA ₁ + ENB ₁	EN A ₁ : 0.365–5	24 h	Synergistic	at low, medium and high cytotoxicity levels (IC $_{25,50,75}$)	[189]
Chinese hamster ovary cells: CHO-K1	ENB ₁ : 0.625–5	2411	Additive	at very high cytotoxicity levels (IC 90)	[100]
ENA ₁ + ENB ₁ Human epithelial colorectal adenocarcinoma cells: Caco-2	EN A ₁ : 0.365–5 ENB ₁ : 0.625–5	24 h	Additive Synergistic	at the lowest fraction affected (IC5) at medium fractions affected (IC25, 50) at the two highest fractions affected (IC75, 90)	[196]
ENB+ENB ₁ Chinese hamster ovary cells: CHO-K1	ENB: 0.365–5 ENB ₁ : 0.625–5	24 h	Additive	at all inhibitory concentration levels (IC $_{\rm 25,\ 50,\ 75,\ 90})$	[189]
ENB+ENB ₁ Human epithelial colorectal adenocarcinoma cells: Caco-2	ENB: 0.365–5 ENB ₁ : 0.625–5	24 h	Antagonistic Additive	at the two lowest fractions affected (IC _{5, 25}) at other fractions affected (IC _{50, 75, 90})	[196]
ENA+ENA1+ENB Chinese hamster ovary cells: CHO-K1	ENA: 0.3125–2.5 ENA ₁ : 0.3125–2.5 ENB: 0.3125–2.5	24 h	Synergistic Additive	at low and medium cytotoxicity levels (IC $_{25, 50}$) at high cytotoxicity levels (IC $_{75, 90}$)	[189]
ENA+ENA ₁ +ENB Human epithelial colorectal adenocarcinoma cells: Caco-2	ENA: 1.25–5 ENA ₁ : 1.25–5 ENB: 1.25–5	24 h	Antagonistic Additive Synergistic	at the lowest fraction affected (IC ₅) at medium fractions affected (IC _{25, 50}) at the two highest fractions affected (IC _{75, 90})	[196]
ENA+ENA ₁ +ENB ₁ Chinese hamster ovary cells: CHO-K1	ENA: 0.3125–2.5 ENA ₁ : 0.3125–2.5 ENB ₁ : 0.3125–2.5	24 h	Synergistic Additive Antagonistic	at low and medium cytotoxicity levels (IC _{25, 50}) at high cytotoxicity level (IC ₇₅) at very high cytotoxicity level (IC ₉₀)	[189]
ENA+ENA ₁ +ENB ₁ Human epithelial colorectal adenocarcinoma cells: Caco-2	ENA: 1.25–5 ENA ₁ : 1.25–5 ENB ₁ : 1.25–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC $_5$) at other fractions affected (IC $_{25}$, $_{50}$, $_{75}$, $_{90}$)	[196]
ENA+ENB+ENB ₁ Chinese hamster ovary cells: CHO-K1	ENA: 0.3125–2.5 ENB: 0.3125–2.5 ENB ₁ : 0.3125–2.5	24 h	Synergistic Additive	at low and medium cytotoxicity levels (IC _{25, 50}) at high cytotoxicity levels (IC _{75, 90})	[189]
ENA+ENB+ENB₁ Human epithelial colorectal adenocarcinoma cells: Caco-2	ENA: 1.25–5 ENB: 1.25–5 ENB ₁ : 1.25–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC $_5$) at other fractions affected (IC $_{25}$, $_{50}$, $_{75}$, $_{90}$)	[196]
ENA ₁ +ENB+ENB ₁ Chinese hamster ovary cells: CHO-K1	ENA ₁ : 0.3125–2.5 ENB: 0.3125–2.5 ENB ₁ : 0.3125–2.5	24 h	Synergistic Additive Antagonistic	at low cytotoxicity level (IC ₂₅) at medium and high cytotoxicity levels (IC _{50,75}) at very high cytotoxicity level (IC ₉₀)	[189]
EN A ₁ +ENB+ENB ₁ Human epithelial colorectal adenocarcinoma cells: Caco-2	ENA ₁ : 1.25–5 ENB: 1.25–5 ENB ₁ : 1.25–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC ₅) at other fractions affected (IC ₂₅ , $_{50}$, $_{75}$, $_{90}$)	[196]
ENA+ENA₁+ENB+ENB₁ Human epithelial colorectal adenocarcinoma cells: Caco-2	ENA: 1.25–5 ENA ₁ : 1.25–5 ENB: 1.25–5 ENB: 1.25–5	24 h	Antagonistic Additive	at the lowest fraction affected (IC ₅) at other fractions affected (IC ₂₅ , $_{50}$, $_{75}$, $_{90}$)	[196]

Abbreviations: deoxynivalenol (DON); 3-acetyldeoxynivalenol (3-ADON); 15-acetyldeoxynivalenol (15-ADON); nivalenol (NIV); fusarenone-X (FUS-X); T-2 toxin (T-2); beauvericin (BEA); fumonisin B1 (FB1); zearalenone (ZEA); α-zearalenol (α-ZOL); β-zearalenol (β-ZOL); enniatins A, A1, B, B1 (ENA, ENA1,ENB, ENB1)



Figure 5. Characterization of the interaction between mycotoxins.

A deeper view of the different interactions between mycotoxins can be found in the review by Grenier and Oswald [6], in which three types of synergism are presented and two kinds of antagonistic effects are itemized.

4.2. Main Experimental Designs for Studying Mycotoxin Interactions

Several experimental designs can be used for studying mycotoxin interactions. Klarić *et al.* [152] briefly described the main mathematical designs used for this purpose: central composite design (CCD), full factorial design, ray design, isobolographic analyses/combination index, and the arithmetic definition of additivity. Some authors used other approaches such as the interaction index V [159] and the coefficient of drug interaction (CDI) [210] to characterize the type of interaction. The aim of all these experimental designs is to predict combined mycotoxin effects based on the comparison between the observed and expected effects of a mycotoxin mixture. The most used models are described hereafter.

The main approach is the one applied by Weber *et al.* [212] and used in more than 30% of the 58 studies. This method is based on the comparison of theoretical expected values calculated on the basis of mono-exposure experiment results with the observed values obtained from co-exposure experiment. In the case of binary mycotoxin combination exposure, the expected cell viability value is calculated as follows:

Cell viability expected value for Mycotoxin1 + Mycotoxin2 (%) = mean cell viability for Mycotoxin1 (%) + mean cell viability for Mycotoxin2 (%) - mean control condition (100%) The expected standard error of mean (S.E.M.) is calculated as follows:

S.E.M. expected for Mycotoxin1 + Mycotoxin2
=
$$[(S.E.M. \text{ for Mycotoxin1}) + (S.E.M. \text{ for Mycotoxin2})]^{1/2}$$

Combined cytotoxic effects are determined by comparison between each expected value and the corresponding measured mean value obtained from co-exposure experiments, often using an unpaired *t*-test. No statistical difference between expected and measured cell viability values is interpreted as an additive effect on cell viability reduction, whereas a synergistic or antagonistic effects are determined if the measured cell viability values are respectively significantly below or above the expected values.

The second most used method, applied in 22% of the analyzed articles, is the combination index-isobologram analysis also known as the Chou-Talalay method [213,214], derived from the Median-effect principle and originally used for analyzing drug combination effects. In isobolographic analyses, the isoeffective points can be interpolated from the results (of cell viability tests for example) and used to plot the isobologram, represented by a line joining equally effective doses (Figure 6). In this type of graph, the additive effect follows the diagonal line between the effective concentrations of each single mycotoxin. If the measured combined effect of two mycotoxins is above or below the diagonal line, it indicates an antagonist or a synergistic effect of the combination respectively. Chou introduced the term "combination index" (CI) to quantify the degree of mycotoxin interaction between two or more mycotoxins [213]. The CI method is often used to analyze the mycotoxin interaction, and the CI values are calculated as follows:

$$(CI)_{x}^{n} = \sum_{j=1}^{n} \frac{(D)_{j}}{(D_{x})_{j}}$$

where $(CI)_x^n$ is the CI for *n* mycotoxins at x% cell viability inhibition, $(D)_j$ is the doses of *n* toxins that exerts x% inhibition in combination, $(D_x)_j$ is the doses of each of *n* mycotoxins alone that exerts x% inhibition.



Figure 6. Isobologram illustrating the antagonist effect of two mycotoxins for reaching x% of cell viability inhibition.

A CI near 1 indicates an additive effect, CI < 1 indicates synergism, and CI > 1 indicates antagonism of the combined mycotoxins. The CI-isobologram method allows not only for determination of the type of interaction but also of its magnitude. This is presented in more detail by Ruiz *et al.* [197] and others.

Only four authors used a CCD including a full or fractional factorial design for mixtures [173,188,202,209]. Briefly, the CCD is used in order to minimize the number of possible toxin combinations from all possible combinations of every concentration (*m* concentrations) of each

toxin (*k* toxins) = mk, to n = 2k/2 cube points + 2k star points + 1 center point. Then, a full or fractional factorial design is applied to detect interactions at various mixture ratios [173]. Nevertheless, when the number of mycotoxins increases and the number of design points needed to study the toxin mixtures becomes too high, another alternative is the ray design providing constant mixture ratios and thus reducing the amount of experimental efforts. Only one author group out of the 58 analyzed articles used this design [203].

The sample number is not a limiting factor regardless of the considered model. However, the simplest and the most intuitive mathematical design seems to be the arithmetic definition of additivity and applied by Weber *et al.* [212] because it is based on a simple additivity of the individual mycotoxin toxicological effect values. Nevertheless, this definition of the combined effects, namely simply defined by the sum of single effects, is questionable, and the example of the combined effect study of the sum of several doses of the same mycotoxin, which cannot be synergistic or antagonistic, highlights this point.

It could be interesting to use different statistical models to analyze a specific mycotoxin mixture under identical exposure conditions to verify the similarity of the results and conclusions, and thus, to determine if it is necessary to standardize the method.

4.3. In Vitro Interactions Between Fusariotoxins

The global results from *in vitro* cell viability studies concerning fusariotoxin mixtures (subdivided in as follows: TCT mixtures; TCT + fusariotoxins and other fusariotoxins mixtures) are presented in Table 2. According to the analyzed studies, trichothecenes as well as other fusariotoxins (ZEA, FUM and emerging mycotoxins), individually and in combination, inhibit cell viability *in vitro*.

Alassane-Kpembi et al. [154,155] showed that combination of DON and its acetylated derivatives 3-DON and/or 15-ADON mainly resulted in synergistic cytotoxicity on porcine IPEC-1 and human Caco-2 cells, and particularly at low inhibitory concentration levels (Inhibitory Concentrations from 10% to 30% = IC_{10} - IC_{30}) on Caco-2. Additive effects were observed at higher doses (IC_{50}). Concerning one of the most studied mixtures, DON+NIV, multiple effects have been observed. The same authors showed synergistic effects on Caco-2 and IPEC-1 (between 0.2 and 15 μ M) [154,155], while Wan *et al.* observed antagonistic effect at 0.5 µM and synergism at 2 µM on porcine IPEC-J2 [209], whereas Marzocco et al. described additivity at medium cytotoxicity level (IC₅₀, or 15 μ M) on murine J774A.1 [194]. DON+FX resulted in synergistic cytotoxicity on Caco-2 and antagonistic effect on IPEC-1, whereas NIV+FX resulted in synergistic effect at low cytotoxicity levels (IC_{10} - IC_{20}) and additivity at higher inhibitory concentration levels (IC_{30} – IC_{50}) on Caco-2 and only additivity on IPEC-1 (IC₁₀–IC₈₀) [154,155]. For DON+T2, antagonism was observed with acute exposure (24 to 72 h) on Chinese hamster CHO-K1 and monkey Vero cells [197,198], and additivity was reported with human progenitors CFU-GM with 14 days of exposure [166]. Therefore, even if a global observation of synergistic toxicity was often observed at low cytotoxicity doses (IC_{10} – IC_{30}), trichothecene mixtures resulted in various cytotoxicity effects which seem to depend on the studied mycotoxin combination, the used cell model, the time of exposure and the tested concentration.

Several authors were interested in mixtures of TCT and other fusariotoxins, such as FB1, ZEA, and the emerging mycotoxin BEA. Again, the conclusions of the different authors and studies were species- and organ-dependent: Ruiz *et al.* observed antagonistic effects on hamster CHO-K1 and monkey Vero cells with DON+BEA co-exposure [197,198], whereas Ficheux *et al.* showed synergism on human CFU-GM [166]. Ruiz *et al.* also studied T2+BEA and showed opposite cytotoxic effect on CHO-K1 and Vero cells (synergism and antagonism respectively) despite the similar mycotoxin doses, the same time of exposure (24 to 72 h) and the same used assessment to measure cell viability (neutral red assay) [197,198]. These opposite observations highlight the complexity of the mycotoxin interactions, with the influence of the used cell models (studied species and targeted organs).

Regarding the ternary mixture DON+T2+BEA studied by Ruiz *et al.*, the effects were the same as those observed for T2+BEA on CHO-K1 and Vero cells [197,198]. Ficheux *et al.* [166] as well as Wan *et al.* [209] observed antagonistic effects with DON+FB1 on CFU-GM and IPEC-J2 at low

concentrations, respectively (less than 0.5 μ M DON and 20 μ M FB1), whereas Kouadio *et al.* showed additivity on Caco-2 at similar doses [187]. Wan *et al.* also observed the same effect on IPEC-J2 with NIV+FB1 and DON+NIV+FB1, that DON+FB1 (namely antagonism at the lowest dose (0.5 μ M DON and NIV, and 20 μ M FB1) and synergism at the highest dose (2 μ M DON and NIV, and 40 μ M FB1)) [209]. Concerning DON+ZEA, another mixture of interest, Kouadio *et al.* [187] as well as Ficheux *et al.* [166] showed additive cytotoxicity on Caco-2 and CFU-GM respectively, whereas Wan *et al.* [209] and Bensassi *et al.* [156] observed antagonism on IPEC-J2 and human HCT116 cells respectively, like for NIV+ZEA and DON+NIV+ZEA [209]. Ficheux *et al.* [166] and Bouaziz *et al.* [158] showed the additivity of T2+ZEA on CFU-GM and Vero cells. Wan *et al.* also studied DON+ZEA+FB1, NIV+ZEA+FB1 and DON+NIV+ZEA+FB1 mixtures, and observed the same effects, namely antagonism at the lowest dose (0.5 μ M DON and NIV, 10 μ M ZEA and 20 μ M FB1)and synergism at the highest dose (2 μ M DON and NIV, and 40 μ M ZEA and FB1), as all the other mixtures they studied on IPEC-J2 [209], whereas Kouadio *et al.* showed additivity for DON+ZEA+FB1 on Caco-2 [187].

Concerning the mixtures involving ZEA, FB1 and emerging mycotoxins such as BEA and ENs, a major part presented antagonistic or additive cytotoxic effects. In particular, ZEA and its derivatives α - and β -zearalenol (α -ZOL and β -ZOL) in binary and ternary mixtures were studied by Wang *et al.* [208] and Tatay *et al.* [204]. Wang *et al.* showed mainly an antagonistic effect of ZEA+ α -ZOL on HepG2 [208], whereas Tatay et al. mostly observed additivity between ZEA and its derivatives on CHO-K1 [204]. Regarding ZEA+FB1, Kouadio et al. [187] and Wan et al. [209] observed antagonistic effects on Caco-2 and IPEC-J2. Klarić et al. showed additivity of FB1+BEA at the lowest concentration (about 0.06 µM BEA and FB1) and synergism at the highest dose (about 6 µM BEA and FB1) on PK15 cells [180]. Concerning emerging mycotoxin mixtures, Ficheux et al. studied BEA+ENB and observed additivity on CFU-GM after 14 days [166]. Finally, several authors [189,196] studied binary, ternary, and quaternary EN mixtures (ENA, ENA1, ENB, and ENB1) and in similar concentrations, with the same cell viability assessment and time of exposure (MTT assay, during 24 h). Globally, Lu et al. observed synergistic effects at low cytotoxicity levels (IC₂₅) and additivity at medium and high inhibitory concentration levels (IC₅₀–IC₉₀) on CHO-K1 [189,196], whereas Prosperini et al. indicated antagonism at low cytotoxicity levels (IC₅–IC₂₅) and additivity at medium and high inhibitory concentration levels $(IC_{50}-IC_{90})$ on Caco-2 cells [189,196], highlighting, once again, the influence, among other, of the type of cell used.

Thus, observed effects are not necessarily dose- and time-dependent. For example, the studies of ENA+ENA1 combined effects by Lu *et al.* and Prosperini *et al.* showed opposite conclusions on CHO-K1 and Caco-2 cells respectively, after 24 h exposure and at the same ENA and ENA1 concentrations [189,196]. Moreover, for a same cell model, interspecies and intraspecies sensitivity depends on tested mixtures. For example, concerning intestinal epithelial cells, exposure effect to DON+NIV were antagonist at low doses ($0.5-2 \mu$ M) for IPEC-J2 (porcine jejunal epithelial cells) and synergistic for IPEC-1 (mix of porcine jejunal and ileal epithelial cells) and human Caco-2 cells. Another observation is for a model cell culture like Caco-2 for example, the number of mycotoxins tested in mixtures could not be predictive of a potential additive or synergistic effect. For example, DON+FX as well as DON+NIV and NIV+FX led to synergistic effect but DON+FX+NIV showed antagonistic effect [155].

Currently, the mycotoxin toxicological combined effects are unpredictable based on their individual effects, despite an increasing number of co-exposure studies.

5. Conclusion

Mycotoxins are present in a large range of feed and food, all over the world, in different concentrations, mainly depending on mould genetics and physiology, outdoor and indoor environment and climate changes. Even if certain mycotoxins often occur together (e.g., AFs+OTA, AFs+FUM or DON+ZEA), an infinity of mixtures may be found. Therefore, combined toxicity effects are very hard to predict. In addition to being influenced by the type of mycotoxin mixtures and their concentrations,

combined toxicity effects depend on the experimental model design: type of cells exposed, time of exposure, ratio used for each mycotoxin in the mixture, endpoints and tests used, as well as chosen statistical model aspects. In general, most of the mycotoxin mixtures lead to additive or synergistic effects, highlighting a significant threat to human and animal health. Moreover, most studies have been carried out over less than three days, at concentrations above the legal limits. There is therefore a lack of data about chronic exposure at sub-toxic mycotoxin concentrations, closer to real food and feed consumption habits. Through a large panel of mycotoxin contamination studies in food and feed around the world, this review constitutes a strong basis of work, allowing for each continent to have an overview of the multicontaminations and to focus on these ones. Diverse publications already showed important combined effects but more studies about relevant mycotoxin combinations should be carried out and especially should be taken into account by the current regulations which only consider so-far mono-exposure data. Finally, the observed diversity of the possible methodological approaches useable (cell models, studied parameters, time and dose exposure, mathematical tools) raises the question of the need for method standardization at an international level allowing for easier data comparison.

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Abbreviations

The following abbreviations are used in this manuscript:

3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
α-ZOL	α-zearalenol
β-ZOL	β-zearalenol
AFs	aflatoxins
BEA	beauvericine
DAS	diacetoxyscirpenol
DON	deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
ENA, ENA1,ENB, ENB1	enniatins A, A1, B, B1
ENs	enniatins
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FB1, FB2, FB3	fumonisin B1, B2, B3
FUM	fumonisins
FUS-X	fusarenone-X
IC	inhibitory concentration
HT-2	HT-2 toxin
MON	moniliformin
NIV	nivalenol
OTA	ochratoxin A
TCTs	trichothecenes
T-2	T-2 toxin
ZEA	zearalenone
WHO	World Health Organization

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Dans cette étude, il a pu être souligné que, parmi les milliers de métabolites secondaires produits par les champignons filamenteux, moins d'une dizaine de mycotoxines sont réglementées à l'heure actuelle. De plus, même la réglementation européenne, une des plus strictes au monde en matière de produits et mycotoxines concernés ainsi que de concentrations autorisées, ne tient pas encore compte des effets combinés des mycotoxines. Il existe toutefois quelques recommandations concernant les teneurs pour certains mélanges de mycotoxines d'une même famille comme c'est le cas pour T-2/HT-2 (2013/165/UE) ou des règlementations comme pour les FUM (EC) No 856/2005. Par ailleurs, concernant les études de multi-contamination, plus de 50% des données sont issues d'échantillons à base de céréales et provenant de l'agriculture européenne. Parmi les 127 mélanges de mycotoxines identifiés dans les denrées alimentaires par différents auteurs, DON+ZEA correspond à l'un plus retrouvés dans les régions tempérées du globe. Enfin, parmi les 58 études in vitro s'intéressant aux effets combinés des mycotoxines, et publiées entre 1980 et 2015, le mélange le plus étudié est OTA+CIT (dans près de 30% des publications), et les modèles cellulaires les plus utilisés sont des modèles rénaux (les modèles cellulaires ciblés étant dépendants de l'organe cible de la mycotoxine étudiée, ce qui explique le choix des modèles rénaux pour l'étude de l'OTA). La viabilité cellulaire reste un des paramètres les plus étudiés dans ce type d'étude, essentiellement mené sur des temps d'exposition très courts (moins de 72h) avec des effets majoritairement additifs et synergiques observés en prenant en considération toutes les espèces, les modèles cellulaires et les mélanges de mycotoxines étudiés. Ainsi, cette publication a permis de mettre en évidence les mélanges majeurs de mycotoxines retrouvés dans l'alimentation en fonction des différents continents, ce qui représente une source de données intéressante pour les différents pays afin de mettre en œuvre des analyses toxicologiques de multi-contaminations plus ciblées. Enfin, cet article souligne l'importance de mener des études in vitro sur des temps d'exposition plus longs et à des concentrations plus faibles et donc plus proches des conditions réelles d'exposition des consommateurs, la nature des effets toxicologiques étant, en partie, dépendante de la durée d'exposition et de la dose testée. Par ailleurs, depuis la publication de cette revue (c'est-à-dire depuis janvier 2016), près de 25 articles s'intéressant aux effets des mélanges de mycotoxines sur des modèles cellulaires in vitro ont été publiés, ce qui représente environ 30% des études sur cette thématique recensées depuis 1980, confirmant l'intérêt croissant de la communauté scientifique pour ce sujet. Les mélanges de mycotoxines, les modèles d'étude ainsi que les effets biologiques rapportés dans ces récentes publications ont été résumés dans le Tableau 1.

Tableau 1 : Effets toxicologiques combinés *in vitro* des mycotoxines observés et publiés depuis 2016 (classés chronologiquement par date de publication).

Mélange de mycotoxines	Modèle cellulaire	Concentration testée	Durée d'exposition	Paramètres étudiés	Principales conclusions obtenues sur les effets combinés observés	Modèles mathématiques	Références
α-ZOL+ β-ZOL α-ZOL+ DON β-ZOL+DON	Cellules bovines de la granulosa GC	α-ZOL : 0,09-3,1 μM β-ZOL : 0,31-31 μM DON : 0,1-3,3 μM	48h	Cytotoxicité et stéroïdogenèse	Additivité Additivité Additivité	Comparaison statistique des données à partir des effets uniques et combinés	(Pizzo et al., 2016)
AOH+DON AOH+ENN B DON+ENN B AOH+DON+ENN B	Cellules d'adénocarcinome du côlon humain Caco-2	AOH : 1,85-30 μM DON : 0,312-5 μM ENN B : 0,312-5 μM	24, 48 et 72h	Cytotoxicité	Synergisme/additivité Synergisme/additivité Antagonisme/additivité Antagonisme/additivité	<i>Combination index</i> (CI) -isobologramme (Chou, 2006; Chou and Talalay, 1984)	(Fernández- Blanco et al., 2016)
TeA+ENN B TeA+ZEA TeA+DON TeA+NIV TeA+NIV TeA+AURO ENN B+ZEA ENN B+DON ENN B+DON ENN B+NIV ENN B+AURO ZEA+DON ZEA+NIV ZEA+AURO DON+NIV DON+AURO NIV+AURO	Cellules d'adénocarcinome du côlon humain Caco-2	TeA : 10-250 μM AURO : 0,1-5 μM DON : 0,1-10 μM ENN B : 1,5-5 μM NIV : 0,1-10 μM ZEA : 10-50 μM	24h	Cytotoxicité	Additivité à la plus faible doses pour tous les mélanges Additivité à la plus fort dose pour les mélanges comprenant AURO et antagonisme pour tous les autres mélanges	CI -isobologramme + Model of independant joint action (IA) (Bliss, 1939)	(Vejdovszky et al., 2016)

DON+ZEA	Cellules de Leydig MA-10	DON : 0,05-0,25 μM ZEA : 10-30 μM	24h	Cytotoxicité et stéroïdogenèse	Antagonisme	Comparaison statistique des données à partir des effets uniques et combinés	(Savard et al., 2016)
BEA+PAT BEA+STE PAT+STE BEA+PAT+STE	Cellules ovariennes de hamster CHO-K1	BEA : 0,156-1,25 μM PAT : 0,049-0,39 μM STE : 0,78-6,25 μM	24, 48 et 72h	Cytotoxicité	Synergisme à faibles doses et additivité à plus fortes doses	CI -isobologramme	(Zouaoui et al., 2016)
BEA+DON BEA+FB1	Cellules d'adénocarcinome du côlon humain Caco-2	BEA : 1,5 μM DON : 3,5 μM FB1 : 1,5 μM	1, 2 et 24h	Résistance trans-épithéliale (TEER) et réponse inflammatoire	Effets du mélange similaires au DON seul sur la TEER et la réponse inflammatoire Effet plus important du mélange sur la TEER que BEA et FB1 seules mais pas sur la réponse inflammatoire	Comparaison statistique des données à partir des effets uniques et combinés	(Albonico et al., 2016a)
β-ZOL+FB1 β-ZOL+FB1 β-ZOL+DON+FB1	Cellules bovines de la granulosa GC	β-ZOL : 93,6 pM DON : 337,8 pM FB1 : 41,6 et 138,7 pM	48h	Cytotoxicité et stéroïdogenèse	Pas d'effet observé sur la viabilité mais effet plus important sur la stéroïdogenèse Pas d'effet observé sur la viabilité et la stéroïdogenèse Pas d'effet observé sur la viabilité et la stéroïdogenèse	Comparaison statistique des données à partir des effets uniques et combinés	(Albonico et al., 2016b)
α-ZOL+FB1 β-ZOL+FB1	Cellules bovines de la granulosa GC	α-ZOL : 15,6 μΜ β-ZOL : 15,6 μΜ FB1 : 6 ,9 μΜ	48h	Cytotoxicité et stéroïdogenèse	Effet des mélanges sur la viabilité et la stéroïdogenèse pas significativement différent des toxines seules		

3-ADON+15-ADON 3-ADON+AOH 15-ADON+AOH 3-ADON +15-ADON+AOH	Cellules d'hépatoblastome humain HepG2	3-ADON : 0,2-1,5 μM 15-ADON : 0,2-1,5 μM AOH : 3,2-24 μM	24, 48 et 72h	Cytotoxicité	Synergisme (CI ₂₅ -90) Synergisme (CI ₂₅ -90) Synergisme à 24h (CI ₂₅ -90), additivité (CI ₂₅ -50) et antagonisme à 48h (CI ₇₅ -90) et additivité à 72h (CI ₂₅ -90) Synergisme (CI ₂₅ -90)	CI -isobologramme	(Juan-García et al., 2016)
DON+ZEA	Cellules rénales embryonnaires humaines HEK-293 (exprimant des protéines fluorescentes)	DON : 1,69-202,7 nM ZEA : 3,14-377,4 nM	8h	Expression de biomarqueurs fluroescents	Effet synergique de ZEA en mélange sur la toxicité de DON	Comparaison statistique des données à partir des effets uniques et combinés	(Ji et al., 2016a)
DON+ZEA	Macrophages murins ANA-1	DON : 3,37 μM ZEA : 25 μM	24h	Métabolome	Réponse métabolomique du mélange présentant des caractéristiques propres (+ inhibition et/ou amplification de certains effets induits par les toxines seules)	Comparaison statistique des données à partir des effets uniques et combinés	(Ji et al., 2016b)
СІТ+ОТА	Levure Saccharomyces cerevisiae (mutant pdr5)	CIT : 400 μM ΟΤΑ : 248 μM	30 min	Transciptome	Réponse génomique du mélange combinant les caractéristiques spécifiques à chaque mycotoxine séparément	Comparaison statistique des données à partir des effets uniques et combinés	(Vanacloig- Pedros et al., 2016)
CIT+OTA 3-ADON+15-ADON +DON α-ZOL+β-ZOL+ZEA	Cellules ostéoblastes humaines U-2 OS (transfectées avec les récepteurs humains	Concentrations non- cytotoxiques	24h et 48h	Activité endocrinienne	Effets des mélanges proches des effets théoriques prédits par le modèle CA (additivité)	Concentration addition model (CA) (Loewe and Muischnek, 1926)	(Demaegdt et al., 2016)

3-ADON+15-DON	TRβ ou PPARx2)						
+DON+FUS-X+NIV	Cellules humaines						
3-ADON+15-ADON	cancéreuses du sein						
+DON+a-ZOL	MCF-7 (récepteur						
+β-ZOL+ZEA	endogène ER : MMV-						
	Luc) et T47-D						
	(récepteur humain						
	AR : TARM-Luc)						
α-ZOL+AFM1					Antagonisme (CI ₂₅₋₉₀)		
α-ΖΟL+ΟΤΑ					Synergisme/additivité (CI25-50)		
					puis antagonisme (CI75-90)		
a-ZOL+ZEA					Antagonisme/additivité (CI25-50)		
					puis synergisme (CI75-90)		
					Synergisme/additivité (CI25-75)		
AFM1+OTA					puis antagonisme (CI ₉₀)		
					Additivité (CI ₂₅) puis		
AFM1+ZEA	Cellules	α-ZOL : 11,25-45 μM			antagonisme (CI ₅₀₋₇₅)		
	d'adénocarcinome du	AFM1 : 2,5-10 μM	24h	Cytotoxicité	Synergisme (CI ₂₅₋₅₀) puis	CL-isobologramme	(Gao et al.,
OTA+ZEA	côlon humain Caco-2	ΟΤΑ : 3,75-15 μΜ	2-11	Cytotoxicite	antagonisme (CI ₇₅₋₉₀)	er isobologramme	2016)
	colon numani Caco-2	ZEA : 7,5-30 μM			Additivité (CI ₂₅₋₅₀) puis		
αZOL+AFM1+OTA					antagonisme (CI ₇₅₋₉₀)		
					Synergisme (CI ₂₅) puis		
αZOL+AFM1+ZEA					antagonisme (CI ₅₀₋₉₀)		
					Synergisme/additivité (CI ₂₅₋₅₀)		
aZOL+OTA+ZEA					puis antagonisme (CI75-90)		
					Synergisme/additivité (CI25-50)		
AFM1+OTA+ZEA					puis antagonisme (CI75-90)		
					Synergisme (CI ₂₅₋₅₀) puis		

aZOL+AFM1					antagonisme (CI ₇₅₋₉₀)		
+OTA+ZEA							
α-ZOL+ZEA OTA+ZEA	Cellules d'hépatoblastome humain HepG2	α-ZOL : 0, 15 et 30 μM OTA : 0, 6 et 12 μM ZEA : 0, 30 et 60 μM	48h	Cytotoxicité	Antagonisme à faibles doses et synergismes à plus fortes doses Antagonisme	Plan factoriel complet (Full Factoriel Design)	(Zheng et al., 2016)
AOH+DON AOH+ZEA DON+ZEA AOH+DON+ZEA	Monocytes humains THP-1	AOH : 0,875-7 μM DON : 0,025-0,2 μM ZEA : 1,25-10 μM	48h	Différenciation cellulaire	Additivité Synergisme à faibles doses et additivité à plus fortes doses Additivité Additivité	Modèles IA, CA et isobologramme	(Solhaug et al., 2016)
DON+NIV	Explants intestinaux porcins	DON : 0-40 μM NIV : 0-40 μM	5h	Réponse inflammatoire	Synergisme	CI -isobologramme	(Alassane- Kpembi et al., 2016)
AFB1+FB1	Hépatocytes de rats BRL-3A	AFB1 : 20 μM FB1 : 30 μM	48h	Cycle cellulaire, apoptose, mortalité cellulaire, stress oxydatif et activité du CYP1A	Effet plus important du mélange sur l'apoptose, la mortalité cellulaire, le stress oxydatif et l'par rapport aux toxines seules, mais effet identique sur le cycle cellulaire	Comparaison statistique des données à partir des effets uniques et combinés	(Mary et al., 2017)
АОН+АТХ П	Cellules d'hépatoblastome humain HepG2 Cellules d'adénocarcinome du côlon humain HT29 Cellules épithéliales	AOH : 5-200 μM ATX II : 0,5-20 μM	24h	Cytotoxicité	Additivité aux faibles doses et antagonisme à plus fortes doses	ΙΑ	(Vejdovszky et al., 2017b)

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CIT+MPA CIT+OTA CIT+PA CIT+PAT MPA+OTA MPA+PA MPA+PA OTA+PAT OTA+PAT PA+PAT	Macrophages bovins BoMacs	CIT : 13,18-52,72 μM MPA : 0,125-0,50 μM OTA : 2,23-8,91 μM PAT : 0,08-0,32 μM PA : 0,975-13,9 μM (0,25*CI ₂₅ -CI ₂₅)	48h	Cytotoxicité	Synergisme $(0.25*CI_{25})$ - antagonisme $(0.5*CI_{25} \text{ et } CI_{25})$ Synergisme $(0,5*CI_{25} \text{ et } CI_{25})$ Antagonisme $(0.5*CI_{25} \text{ et } CI_{25})$ Antagonisme $(0.5*CI_{25})$ Synergisme $(0,25*CI_{25}, 0,5*CI_{25}, 0,5*CI_{25}, 0,5*CI_{25})$ Synergisme ou antagonisme selon le modèle mathématique Antagonisme $(0,25*CI_{25}, 0,5*CI_{25}, 0,5*CI_{25})$ Synergisme $(0.25*CI_{25}, 0,5*CI_{25}, 0,5*CI_{25})$ Synergisme $(0.25*CI_{25}, 0,5*CI_{25})$ Synergisme $(0.25*CI_{25}, 0,5*CI_{25})$ Synergisme $(0,5*CI_{25})$ - antagonisme (CI_{25})	IA + CA	(Oh et al., 2017)
α-ZOL+AOH AOH+ZEA	Cellules humaines d'adénocarcinome de l'endomètre Ishikawa	α-ZOL : 1 pM-10µM ZEA : 10 pM-10µM AOH : 50 nM-10µM	48h	Cytotoxicité et réponse œstrogénique	Synergisme	CI -isobologramme	(Vejdovszky et al., 2017a)
FB1+BEA	Cellules bovines de la granulosa GC	FB1 : 0,5-6 μM BEA : 3 μM	48h	Cytotoxicité et stéroïdogenèse	Effets du mélange similaires aux toxines seules (additivité)	Comparaison statistique des données à partir des effets uniques et combinés	(Albonico et al., 2017)
15-ADON+DON 15-ADON+FUS-X 15-ADON+NIV DON+NIV DON+FUS-X FUS-X+NIV	Cellules épithéliales gastriques humaines GES-1	15-ADON : 1,11-17,7 μM DON : 1,27-20,25 μM FUS-X : 0,35-5,65 μM NIV : 0,4-6,4 μM	24h	Cytotoxicité	Synergisme (CI ₁₀ -CI ₇₀) Antagonisme (CI ₂₀ -CI ₉₀) Antagonisme (CI ₁₀ -CI ₉₀) Synergisme (CI ₁₀ -CI ₈₀) Synergisme (CI ₁₀ -CI ₄₀) Synergisme (CI ₁₀ -CI ₉₀)	CI -isobologramme	(Yang et al., 2017)
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AFB1+DON AFB1+ZEA DON+ZEA AFB1+DON+ZEA	Cellules d'hépatoblastome humain HepG2 Macrophages murins RAW 264.7	AFB1 : 0,08-16 μM DON : 0,0027-2 μM ZEA : 0,28-37,7 μM	48h	Cytotoxicité	Additivité/synergisme Antagonisme Additivité/synergisme Additivité/synergisme	CI -isobologramme	(Zhou et al., 2017)
DON+ZEA	Lymphocytes spléniques porcins	DON : 0,2-5,06 μM ZEA : 0.25-6,28 μM	48h	Apoptose et fonctions antioxydantes	Synergisme	Comparaison statistique des données à partir des effets uniques et combinés	(Ren et al., 2017)

Abréviations : 3-ADON : 3-acétyl-déoxynivalénol ; 15-ADON : 15-acétyl-déoxynivalénol ; α -ZOL : alpha-zéaralénol ; β -ZOL : β -zéaralènol ; AFB1 : aflaoxine B1 ; AFM1 : aflatoxine M1 ; TeA : acide ténuazonique ; AOH : alternariol ; ATX II : altertoxin II ; AURO : aurofusarine ; BEA : beauvericine ; CIT : citrinine ; DON : déoxynivalénol ; ENN B : enniatine B ; FB1 : fumonisine B1 ; MON : moniliformine ; MPA : acide mycophénolique ; NIV : nivalénol ; OTA : ochratoxine A ; PA : acide pénicillique ; PAT : patuline ; STE : sterigmatocystine ; T-2 : toxine T2 ; ZEA : zéaralénone ;

N.B: L'aurofusarine est un métabolite secondaire fongique pour lequel la classification de « mycotoxine » reste discutable en raison de sa faible toxicité pour l'Homme et l'animal.

Parmi ces études, toutes s'intéressent aux effets combinés aigus in vitro des mycotoxines, la plupart du temps sur 24 et/ou 48h d'exposition. Les effets chroniques in vitro des monoexpositions de mycotoxines ne sont d'ailleurs pas plus étudiés. Comme il avait pu être constaté précédemment, le synergisme et/ou l'additivité des mélanges sur la viabilité cellulaire est observé dans la grande majorité de ces études, confirmant l'importance d'étudier ces associations pour une meilleure prise en compte du risque pour le consommateur. Cependant, contrairement à l'observation faite dans la revue, on remarque depuis 2016 que l'utilisation de l'indice de combinaison (« combination index ») est de plus en plus fréquente pour prédire les effets théoriques des mélanges sur la base des effets individuels des mycotoxines qui les composent (modèle établi par Chou (2006) et Chou and Talalay (1984)). D'autre part, les modèles cellulaires les plus utilisés depuis 2016 sont d'origine humaine (plus de 60% des études). En particulier, les modèles cellulaires humains d'origine intestinale et hépatique sont parmi les plus étudiés, avec notamment l'utilisation des lignées Caco-2 (cellules de l'épithélium intestinal humain) et HepG2 (hépatocytes humains) dans 4 des articles cités ci-dessus pour chacun de ces modèles. Par ailleurs, les mycotoxines DON, ZEA et leurs métabolites restent parmi les plus étudiés entre 2016 et 2017. En revanche, le manque de données concernant les mycotoxines émergentes est toujours d'actualité. Enfin, depuis 2016, seuls deux articles recensés ont étudié les effets combinés des mycotoxines via des approches «-omiques» non ciblées, et plus particulièrement via des approches de transcriptomique (Vanacloig-Pedros et al., 2016) et de métabolomique (Ji et al., 2016b).

Partie II : Les fusariotoxines, synthèse bibliographique

De manière générale, les moisissures du genre *Fusarium* sont les espèces les plus problématiques dans les régions tempérées du monde, et plus particulièrement en Europe, en raison de leur prévalence, leur écologie, leur physiologie et de la large gamme de fusariotoxines produites (TCTs, FUMs, ZEA, BEA, FUS-X ou encore MON) (Smith et al., 2016; Yazar and Omurtag, 2008). Cependant, même s'il existe des zones plus fortement concernées par la présence des *Fusarium* spp. et de leurs mycotoxines, les échanges commerciaux contribuent à leur dispersion géographique et les denrées alimentaires contaminées se retrouvent partout à travers le monde.

Dans cette partie, les trois grandes familles de fusariotoxines (TCTs, FUMs et ZEA), qui sont aujourd'hui les mieux caractérisées, et plus particulièrement le DON, le NIV et la toxine T-2 (T2) appartennant à la famille des TCTs, ainsi que la fumonisine B1 (FB1) faisant partie des FUMs, seront présentées. Enfin, la MON fera également l'objet d'un chapitre en tant que fusariotoxine « émergente » fréquemment retrouvée en concomitance avec des fusariotoxines majeures.

1. Historique

1.1. Les trichothécènes

Le terme « trichothécène » provient de la trichothéine, un des premiers métabolites identifiés de cette famille, isolée à partir des filtrats de cultures de *Trichothecium roseum* (Freeman and Morrison, 1949). Aujourd'hui, on dénombre plus de 200 métabolites appartenant aux TCTs, qui peuvent être répartis en 4 groupes en fonction de leurs propriétés physiques et chimiques (groupes A, B, C et D) (Shank et al., 2011). Les TCTs des groupes A et B sont les plus répandus dans l'alimentation, et tout particulièrement la T2 et le diacétoxyscirpénol (DAS) qui appartiennent au groupe A, ainsi que le DON et le NIV qui appartiennent au groupe B. Le DON est d'ailleurs le seul TCT réglementé dans l'alimentation humaine par l'Europe et les Etats-Unis d'Amérique (da Rocha et al., 2014; Smith et al., 2016).

Pour mémoire, le DON a d'abord été isolé au Japon en 1972 et a été nommé « Rd-toxin » (Morooka et al., 1972). Peu de temps après, ce même composé a été isolé du maïs et associé à des vomissements chez les porcs, raison pour laquelle il a été renommé « vomitoxine » (Vesonder et al., 1973). Depuis, de nombreux cas confirmés d'intoxications au DON

induisant notamment un refus d'alimentation chez les porcs ont été observés (Osweiler, 2000). Bien que le DON ne soit pas considéré comme sévèrement toxique pour les animaux, il est considéré comme une des causes majeures en termes de pertes économiques en raison de la réduction des performances des élevages et de l'augmentation de la sensibilité des animaux aux pathogènes (Morgavi and Riley, 2007).

D'autres TCTs, comme la T2, apparaissent beaucoup plus toxiques. Son utilisation (associée à d'autres TCTs) comme arme chimique en Iran, Afghanistan, au Viêtnam et au Laos entre 1975 et 1981 a d'ailleurs été suspectée (Heyndrickx et al., 1984; Rosen and Rosen, 1982). Le gouvernement américain décrivit de fines particules jaunes s'échapper d'avions volant à basse altitude au-dessus des populations rurales locales, ce qui valut à la T2 le nom de « pluie jaune » (Barceloux, 2008).

1.2. Les fumonisines

Les FUMs ont été décrites et caractérisées pour la première fois en 1988 (Bezuidenhout et al., 1988; Gelderblom et al., 1988). Cependant, leurs effets sur de nombreuses espèces animales sont connus depuis longtemps, notamment chez les chevaux, chez lesquels leur ingestion se manifeste par une hépatotoxicité et une nécrose cérébrale. Jusqu'à 28 FUMs réparties en 4 groupes, A, B, C et P, ont jusqu'à aujourd'hui été identifiées (Rheeder et al., 2002; Yazar and Omurtag, 2008). Les fumonisines B1, B2 et B3 (respectivement FB1, FB2 et FB3) sont les principales FUMs retrouvées comme contaminants naturels des céréales (Richard et al., 2003). En particulier, la FB1 est le membre le plus abondamment produit de cette famille de mycotoxines, et représente entre 70 et 80% de la teneur totale en FUMs des cultures de *Fusarium* (Reddy et al., 2010; Rheeder et al., 2002). De plus, il s'agit de la FUM la plus préoccupante d'un point de vue toxicologique.

1.3. La zéaralénone

La première étude reliant la consommation d'aliments contaminés par des moisissures avec l'œstrogénisme chez le cochon date de 1928 (McNutt et al., 1928). Cependant, c'est au cours des années cinquante que la ZEA a été identifiée et reconnue comme étant la cause d'un trouble de la reproduction chez le porc connu sous le nom de vulvovaginite. Anciennement appelée toxine F-2, l'effet œstrogénique de cette mycotoxine est aujourd'hui bien connu. Cependant, la classification de la ZEA comme mycotoxine est contestée car elle présente une faible toxicité aiguë pour l'Homme et l'animal. Ce métabolite est donc plus couramment

classé en tant que « mycœstrogène » (Bennett and Klich, 2003). D'autre part, la caractéristique la plus importante des espèces de *Fusarium* synthétisant la ZEA est leur capacité à produire également des TCTs, ce qui soulève un point important concernant la possible additivité ou le synergisme des effets combinés de ces mycotoxines dans l'étiologie des mycotoxicoses humaines et animales (Richard et al., 2003).

1.4. La moniliformine

Le développement des méthodes d'analyse de plus en plus résolutives au cours des dernières années a permis l'identification de mycotoxines « émergentes », telles que la MON, sur lesquelles peu de données sont disponibles, et qui sont pourtant très présentes dans les céréales, souvent en présence de mycotoxines « majeures » (Escrivá et al., 2015). La MON a été isolée en 1973 à partir d'une culture de *Fusarium moniliforme* (aujourd'hui identifié comme *Fusarium proliferatum*) (Cole et al., 1973), et retrouvée pour la première fois dans du maïs provenant du Transkei, en Afrique du Sud (Thiel et al., 1982).

2. Structure et propriétés physico-chimiques

2.1. Les trichothécènes

Les TCTs appartiennent au groupe des sesquiterpènoïdes qui possèdent un squelette tricyclique, appelé trichothécane, formé par un cyclopentane, un cycle à six chaînons oxygénés et quatre groupements méthyles. Tous les TCTs naturels possèdent une double liaison en C-9,10 ainsi qu'un groupement époxy en C-12,13 caractéristique des 12,13-époxy-trichothécènes (Ueno, 1980, 1985). La structure générale semi-développée des TCTs avec la numérotation des atomes de carbone est disponible en figure 1. Par ailleurs, l'ouverture du cycle 12-13 époxyde conduit à la formation de dérivés inactifs (Ueno, 1980).

Une classification des TCTs en 4 groupes selon leurs propriétés physiques et chimiques a été proposée par Ueno (1977) : une première division des TCTs en deux catégories permet de distinguer les TCTs macrocycliques, qui possèdent un ester macrocyclique, des TCTs non-macrocycliques, présentant un pont ester-éther entre le C-4 et le C-15. De plus, les TCTs non-macrocycliques peuvent être subdivisés en trois groupes : A, B et C, alors que les non-macrocycliques sont communément regroupés sous le nom de TCTs du groupe D. Ces différents groupes sont caractérisés de la façon suivante :

- Les TCTs non-macrocycliques :
- le groupe A, constitué des TCTs qui ont une chaîne latérale de type hydrogène ou ester en position C-8, tels les toxines T-2 et HT-2, ainsi que le DAS ou encore le néosolaniol ;
- le groupe B, composé des TCTs possédant une fonction cétone en C-8, tels que le DON, le NIV et la fusarénone-X (FUS-X);
- le groupe C, comprenant les TCTs qui possèdent un époxyde supplémentaire en C-7/8, comme la crotocine ;
- Les TCTs macrocycliques :
- le groupe D, constitué des TCTs possédant un macrocycle entre le C-4 et el C-15, tels que les verrucarines, les roridines et les satratoxines.



Figure 1 : Structure chimique générale des trichothécènes.

La toxine T2, ou 12,13-epoxytrichothec-9-ene- 3α ,4 β ,8 α ,15-tetraol,4,15-diacetate 8isovalerate, est également connue sous le nom de fusariotoxine T2, 8-isovalerate, isariotoxine ou mycotoxine T-2. Sa formule brute est C₂₄H₃₄O₉ et sa structure chimique est donnée dans le tableau 2. Son poids moléculaire est de 466,52 g/mol et son numéro d'enregistrement unique auprès de la banque de données de Chemical Abstracts Service (CAS) est 21259-20-1.

Le DON, ou trichothec-9-ene-8-one,12,13-epoxy- 3α , 7α ,15-trihydroxy-, a comme autres noms dehydronivalenol, 4-deoxynivalenol, 12,13-epoxy- 3α , 7α ,15-trihydroxy-9-trichothecen-8-one, Rd toxine, spiro[2,5-methano-1-benzoxepin-10,2'-oxirane]trichothec-9-en-8-one dérivé ou encore vomitoxine. Sa formule brute est C₁₅H₂₀O₆ et sa structure chimique est donnée dans le tableau 2. Son poids moléculaire est de 296,32 g/mol et son numéro CAS est 51481-10-8.

Le NIV, ou trichothec-9-en-8-one,12,13-epoxy- 3α , 4α ,7,15-tetrahydroxy, a pour formule brute C₁₅H₂₀O₇. Sa structure chimique est donnée dans le tableau 2. Son poids moléculaire est de 312,32 g/mol et son numéro CAS est 23282-20-4.

Mycotoxine	R 1	R2	R3	R4	R5	
Туре А						
Diacétoxyscirpenol	ОН	∼o ^d ⊂H₃	∼о ^Д сн₃	Н	Н	
Néosolaniol	ОН	∼o ^{CH} ₃	~o [∩] ⊂ _{CH3}	Н	ОН	
Toxine HT-2	ОН	ОН	~o [↓] _{CH3}	Н		
Toxine T-2	ОН	∼o ^{CH} 3	∼о ^Д сн₃	Н		
Type B						
Déoxynivalénol	ОН	Н	OH	OH	=0	
Fusarénone-X	ОН	∼o [⊖] ⊂ _{CH₃}	ОН	ОН	=O	
Nivalénol	ОН	ОН	ОН	OH	=O	
Type C						
Crotocine	Н	о Снз	Н		Epoxyde	
Type D						
Roridine E	н	C15 C4 O O CH ₃ HO	H CH3	Н	Н	
Satratoxine H	н	0 0 H0 H3C			Н	
Verrucarine A	Verrucarine A H H_{H_0}		Н	Н		

Tableau 2 : Structure chimique de certains trichothécènes d'après Shank et al. (2011). Lesgroupes R se réfèrent aux substituants présentés en figure 1.

De manière générale, les TCTs sont très stables : ils restent stables à température ambiante, et ne sont pas détruits lors de la cuisson des aliments, ni dans les conditions de stérilisation, comme celles appliquée au lait (environ 15 min à 118°C) (Vidal et al., 1985). Lors de l'étude de la stabilité de quatre TCTs, le T2, l'HT2, le DON et le NIV, il a été montré que l'acétronitrile était le solvant dans lequel ils étaient le plus stables, aucune dégradation de ces toxines n'ayant été observée pendant 24 mois de stockage à 25°C (Widestrand and Pettersson, 2001). En revanche, le DON, qui est soluble dans l'éthanol, le méthanol, l'acétate d'éthyle ou encore l'eau, peut-être conservé dans l'acétate d'éthyle de façon stable pendant au moins 24 mois uniquement à -18°C. A des températures supérieures (24 mois à 4°C, 12 mois à 25°C), le DON se décompose significativement. Enfin, le NIV est soluble dans les solvants organiques polaires comme dans l'éthanol, le méthanol, l'acétate d'éthyle ou encore le chloroforme mais est cependant faiblement soluble dans l'eau. Comme le DON, il reste stable 24 mois à -18°C dans l'acétate d'éthyle et se dégrade plus rapidement à des températures de stockage plus élevées.

2.2. Les fumonisines

Les FUMs constituent un groupe de mycotoxines chimiquement voisines. La FB1, de formule brute $C_{34}H_{59}O_{15}$ et de masse moléculaire 721,83 g/mol, est le diester en positions 14 et 15 de l'acide 1,2,3-propane tricarboxylique et du 2-amino-12,16-diméthyl-3,5,10,14,15-pentahydroxyéicosane. La FB2 et la FB3, ayant tous deux pour formule brute $C_{34}H_{59}O_{14}$ et pour poids moléculaire 705,8 g/mol, sont respectivement les analogues du désoxy C-10 et du désoxy C-5 de la FB1 (Soriano et al., 2005). La structure semi-développée de la FB1 est donnée en figure 2. Son numéro CAS est 116355-83-0.



Figure 2 : Structure chimique de la fumonisine B1.

Ces composés, fortement hydrophiles du fait des 4 fonctions acides carboxyliques qui les caractérisent, sont solubles dans l'eau et insolubles dans les solvants organiques. D'autre part, les FUMs sont relativement stables à la chaleur. En effet, leur teneur dans les aliments ne semble être significativement réduite que pendant les processus de transformation au cours desquels la température dépasse 150 °C (Bullerman and Bianchini, 2007; Jackson et al., 1996).

2.3. La zéaralénone

La ZEA, de formule brute $C_{18}H_{22}O_5$ et de masse molaire 318,36 g/mol, est la lactone désaturée à fonction cétone de l'acide résorcylique (zéaralane) (Mirocha et al., 1967; Urry et al., 1966). Il s'agit d'un énantiomorphe de l'acide- β -résorcylique-6-(10'-hydroxy-6'-céto-trans-undécnyl)- μ -lactone. Sa structure semi-développée est donnée en figure 3. Son numéro CAS est 17924-92-4.



Figure 3 : Structure chimique de la zéaralénone.

La ZEA est très faiblement soluble dans l'eau et dans l'hexane ; sa solubilité augmente avec la polarité des solvants (Hidy et al., 1977). L'acétate d'éthyle est d'ailleurs le solvant le plus utilisé pour son extraction dans les produits alimentaires. De plus, la ZEA est un composé stable à la fois pendant le stockage/fraisage et le traitement/cuisson des aliments jusqu'à 150°C (Yazar and Omurtag, 2008). Sa dégradation n'a été observée qu'à des températures élevées ou dans des conditions alcalines (Ryu et al., 1999).

2.4. La moniliformine

La MON, de formule brute $C_4H_2O_3$ et de poids moléculaire 98 g/mol, est le sel de potassium ou de sodium du 3-hydroxycyclobut-3-ène-1,2-dione (Betina, 1989). Sa structure semidéveloppée est représentée en figure 4. Son numéro CAS est 31876-38-7.



Figure 4 : Structure chimique de la moniliformine sous la forme de sel de (a) sodium et (b) potassium.

Cette molécule est très soluble dans l'eau grâce à la forte polarité (Cole and Cox, 1981; Sydenham et al., 1996). La MON reste stable dans les procédés alimentaires qui ont lieu dans des conditions neutres ou acides, telle que la cuisson. Cependant, en milieu alcalin, la cuisson peut provoquer une réduction partielle ou complète de cette molécule, en fonction de la température et du temps de cuisson. De plus, la lyophilisation ne modifie pas la stabilité de la MON (Jestoi, 2008).

3. Production

Si toutes les mycotoxines produites par des espèces de *Fusarium* sont couramment appelées « fusariotoxines », telles que les TCTs, les FUMs ou encore la ZEA, elles ne sont pourtant pas toutes exclusivement produites par des *Fusarium* spp. En effet, dans le cas des TCTs par exemple, bien que les *Fusarium* soient les espèces productrices majoritaires, ces mycotoxines peuvent également être sécrétées, entre autres, par des champignons des genres *Myrothecium Phomopsis, Stachybotrys, Trichoderma, Trichotecium, Verticimonosporium* (Bennett and Klich, 2003; da Rocha et al., 2014; Ueno, 1985). Cependant, dans ce paragraphe, seules les espèces appartenant au genre *Fusarium* seront présentées.

La production de mycotoxines (toxicogénèse) dépend étroitement des conditions de croissance des moisissures. En particulier, il a été établi qu'une phase de croissance mycélienne était nécessaire pour qu'il y ait synthèse et excrétion de mycotoxines (Dombrink-Kurtzman and Blackburn, 2005; Kokkonen et al., 2005; Molina and Giannuzzi, 2002). D'une manière générale, la croissance mycélienne dépend principalement des facteurs suivants :

 la nature du substrat : les végétaux riches en glucides et en cellulose, tels que les céréales et les ensilages, constituent les substrats les plus favorables au développement des moisissures toxinogènes ;

- l'activité de l'eau du substrat (a_w) : la quantité d'eau disponible nécessaire à la croissance fongique varie en fonction de l'hygrométrie. En dessous d'une a_w de 0,6, le développement fongique n'est plus possible. Les genres hygrophiles, comprenant les *Fusarium*, se développent à des a_w supérieures à 0,9 (tableau 3);
- la température : chaque espèce est caractérisée par un minimum, un optimum et un maximum de croissance. Les *Fusarium* ont un optimum situé entre 20°C et 25°C, c'est pourquoi on les retrouve essentiellement dans les régions tempérées (tableau 3);
- la présence de gaz dans le substrat : les moisissures étant aérophiles, l'absence d'oxygène limite leur développement.

Tableau 3 : Températures et a_w optimales pour la production de quelques fusariotoxines majeures (Milani, 2013; Paterson and Lima, 2011).

	Températures (°C)	a _w
FUM		
F. proliferatum et F. verticillioides	15-30	0,9-0,995
ZEA		
F. culmorum et F. graminearum	25	0,96
DON		
F. culmorum et F. graminearum	26-30	0,995

3.1. Les trichothécènes

Parmi les espèces productrices de TCTs, on trouve *F. acuminatum, F. armeniacum, F. cerealis, F. culmorum, F. equiseti, F. graminearum, F. incarnatum, F. kyushuense, F. langsethiae, F. lateritium, F. oxysporum, F. poae, F. pseudograminearum, F. sambucinum, F. scirpi, F. solani, F. sporotrichioides et F. tricinctum (Smith et al., 2016; Ueno, 1980). En général, la plupart des espèces, suivant les conditions, peuvent produire plusieurs TCTs (Ueno, 1980).*

La toxine T2 a été isolée pour la première fois en 1968 à partir de *Fusarium tricinctum*. Depuis, il a pu être observé que de nombreuses espèces de *Fusarium* produisaient la T2, telles que *F. acuminatum*, *F. culmorum*, *F. equiseti*, *F. incarnatum*, *F. kyushuense*, *F. poae*, *F. solani* et plus couramment, *F. sporotrichioides* et *F. langsethiae* (D'Mello and Macdonald, 1997; Doohan et al., 2003; Eriksen, 1998; Glenn, 2007; Marin et al., 2013; Thrane et al., 2004; Yazar and Omurtag, 2008).

Le DON a d'abord été isolé d'une souche de *Fusarium roseum* (aujourd'hui renommée *F. graminearum*), qui est d'ailleurs l'une des principales espèces qui le produisent avec *F. culmorum*. Les autres espèces capables de produire du DON sont *F. acuminatum, F. avenaceum, F. cerealis, F. oxysporum, F. pseudograminearum, F. solani, F. sporotrichioides* et *F. tricinctum* (D'Mello and Macdonald, 1997; Eriksen, 1998; Glenn, 2007, 2007; Marin et al., 2013; Ueno, 1980; Yazar and Omurtag, 2008).

Le NIV a pu être isolé d'une souche de *Fusarium nivale* (aujourd'hui connue sous le nom de *F. kyushuense*). Cette mycotoxine est essentiellement produite par *F. cerealis* et *F. poae*, mais peut également être sécrétée par *F. culmorum*, *F. graminearum* et *F. kyushuense* (Eriksen, 1998; Glenn, 2007; Yazar and Omurtag, 2008).

Les contaminations par ces *Fusarium* et leurs mycotoxines peuvent survenir en cours de culture ou lors de la récolte en fonction des conditions de température et d'humidité ainsi que du rapport oxygène/dioxyde de carbone (Betina, 1989). En particulier, la croissance de ces moisissures ainsi que la production de mycotoxines sont fortement stimulées par des épisodes de refroidissement (Cisti et al., 1983). Néanmoins, elles peuvent être neutralisées par l'acidité dans le cas, par exemple, des ensilages. Il arrive également que la contamination survienne en cours de stockage, en conditions humides, avant séchage des grains. C'est notamment le cas du maïs en *cribs* (lieux de stockage permettant un séchage à l'air libre sans apport d'énergie) dont l'installation est propice à une forte humidité (AFSSA, 2009).

3.2. Les fumonisines

Les seuls champignons filamenteux qui produisent des quantités significatives de FUMs sont *F. proliferatum* et *F. verticillioides* (Richard et al., 2003). D'autres espèces sont néanmoins capables de synthétiser des FUMs, telles que *F. anthophilum, F. dlamini, F. fujikuroi, F. globosum, F. napiforme, F. nygamai, F. oxysporum, F. polyphialidicum, F. pseudonygamai, F. sacchari, F. subglutinans et F. thapsinum* (Rheeder et al., 2002).

La sécrétion de FUMs par les différentes espèces de *Fusarium* se produit quasi exclusivement au champ. Il arrive malgré tout qu'elles puissent être produites au cours du stockage si les conditions de température et d'humidité sont favorables (Bryden, 2012). Le stress dû à la sécheresse suivi d'un temps chaud et humide plus tard dans la saison de culture semble être un facteur important dans la production des FUMs par les *Fusarium* (AFSSA, 2009; Richard, 2007).

3.3. La zéaralénone

La ZEA est essentiellement produite par des *Fusarium* au cours de la maturation des grains de céréales (AFSSA, 2009). Les principales espèces productrices correspondent à *F. culmorum*, *F. equiseti* et *F. graminearum*, cette dernière étant la principale espèce responsable des effets œstrogéniques observés chez les animaux d'élevage (Nesic et al., 2014). *F. cerealis, F. incarnatum, F. pseudograminearum, F. sporotrichioides* et *F. verticillioides* sont également capables de produire de la ZEA (Bryden, 2012; da Rocha et al., 2014; Eriksen, 1998; Glenn, 2007; Marin et al., 2013; Nesic et al., 2014; Yazar and Omurtag, 2008).

Ces champignons filamenteux produisent principalement la ZEA sur les céréales au champ, et parfois lors du stockage du maïs en *cribs* ou au cours du maltage de l'orge. Les fourrages ensilés peuvent aussi contenir de la ZEA, produite au moment de la mise en silo lors d'un mauvais tassement de celui-ci par exemple. En règle générale, la contamination des céréales après leur récolte n'arrive que si les bonnes conditions de stockage sont mal respectées. La production de ZEA est favorisée par un taux important d'humidité et de faibles températures, autrement dit, lorsque les conditions climatiques sont mauvaises (intempéries) (AFSSA, 2009; Gajecki, 2002). Par ailleurs, Llorens et al. (2004) ont montré que la production était maximale à 20°C, avec toutefois des variations en fonction des souches. De plus, une étude a mis en évidence que des variations successives de températures augmentaient considérablement la production de ZEA (Eugenio et al., 1970).

3.4. La moniliformine

La principale espèce productrice de MON est *F. proliferatum.* Toutefois, cette mycotoxine est également produite par de nombreuses espèces de *Fusarium* isolées de divers substrats et provenant de différentes régions du monde (Abramson et al., 2001; De Nijs et al., 1996; Fotso et al., 2002; Schütt et al., 1998), parmi lesquelles on trouve *F. acuminatum, F. avenaceum, F. culmorum, F. equiseti, F. fujikuroi, F. napiforme, F. nygamai, F. oxysporum, F. pseudonygamai, F. sporotrichioides, F. subglutinans, F. thapsinum, F. tricinctum et F. verticillioides* (Bryden, 2012; Eriksen, 1998; Glenn, 2007; Jestoi, 2008; Marin et al., 2013; Nesic et al., 2014).

D'autre part, la MON peut être stable plusieurs années dans le sol, et peut être transférée à la culture de la génération suivante (Guzman and Casteel, 1994).

4. Occurrence

Depuis plusieurs années déjà, les mycotoxines majeures font l'objet d'analyses à l'échelle mondiale dans le but de déterminer dans quelles proportions et à quelles concentrations elles sont présentes dans l'alimentation. Depuis 2014, la société BIOMIN publie également les résultats d'une analyse multiple de mycotoxines permettant d'identifier jusqu'à 380 métabolites secondaires fongiques simultanément, comprenant de nombreuses mycotoxines émergentes. Les résultats de la dernière enquête publiée en 2016, obtenus à partir de 16511 échantillons céréaliers destinés à l'alimentation animale (maïs, blé, orge, riz, farine de soja, farine de gluten, grains de distillerie séchés ou encore ensilages) provenant de 81 pays, sont présentés dans cette partie (BIOMIN, 2016).

4.1. Les trichothécènes

Les TCTs contaminent une large gamme de matrices céréalières (avoine, blé, maïs, millet, orge, sarrasin, seigle, riz...), ainsi que les fruits tels que la banane (Smith et al., 2016). D'autre part, toutes les régions du monde sont concernées par les contaminations aux TCTs, les plus touchées étant les zones à climat tempéré d'Amérique du Nord, d'Europe et d'Asie. Cependant, comme pour toutes les mycotoxines, selon les conditions météorologiques, la croissance des champignons productifs de TCTs ainsi que la production de mycotoxines varient considérablement d'une année à l'autre (Bennett and Klich, 2003).

Les céréales les plus contaminées par la T2 sont généralement le blé, le maïs, l'avoine, le seigle, le riz, les fèves et le soja (JECFA, 2002). La plupart des données concernant les niveaux de contamination des céréales par la T2 proviennent d'Europe. Dans le reste du monde, peu de données sont disponibles. On trouve cependant des valeurs de contaminations ponctuelles de certaines céréales. En 2002, les données disponibles sur 8918 échantillons céréaliers provenant essentiellement d'Europe montraient que 11% d'entre eux contenaient de la T2 à des concentrations moyennes variant de 0,1 μ g/kg pour l'orge à 60 μ g/kg pour le blé (JECFA, 2002). La dernière enquête de BIOMIN (BIOMIN, 2016) montre que la T2 semble toujours faiblement présente dans les céréales, avec moins de 10% des échantillons contaminés en Amérique du Nord, en Afrique et au Moyen-Orient, à des concentrations inférieures à 50 μ g/kg en moyenne. Le plus fort pourcentage d'échantillons contaminés et les

plus importantes concentrations ont été recensés en Amérique du Sud, et concernent le blé et le sorgho (55% contiennent de la T2 avec des teneurs de 50 μ g/kg en moyenne).

De manière générale, le DON est souvent retrouvé dans l'orge, le maïs, le seigle, les graines de carthame et le blé (Miller et al., 2001). De plus, il fait partie des mycotoxines les plus retrouvées dans le monde, comme le montrait l'enquête réalisée par Rodrigues and Naehrer (2012) entre 2009 et 2011, dans laquelle près de 60% des 7049 échantillons céréaliers analysés étaient contaminés. En 2016, plus de 70% des échantillons analysés contiennent du DON, à l'exception des échantillons provenant du Moyen-Orient (BIOMIN, 2016). La région du monde dans laquelle les quantités retrouvées de DON sont les plus critiques est l'Amérique du Sud, avec 77% des échantillons de blé et de sorgho contaminés à une concentration moyenne de 2834 μ g/kg et une concentration maximale retrouvée de 49790 μ g/kg. L'Europe semble également être fortement affectée par cette mycotoxine, présente essentiellement dans le blé, l'orge, l'avoine et le triticale, à des concentrations d'en moyenne 1023 μ g/kg, et pouvant atteindre 37640 μ g/kg.

Les teneurs en NIV observées en Asie sont de l'ordre de la dizaine de milliers de μ g/kg alors que dans le reste du monde, le niveau de contamination ne dépasse pas 1000 μ g/kg, voire dans la plupart des cas, est inférieur à 500 μ g/kg (Balzer et al., 2004). Le NIV ne faisant pas partie de l'analyse ciblée menée par BIOMIN, seuls les résultats issus des 1378 échantillons céréaliers analysés *via* l'approche « multiple » et ciblant 380 métabolites fongiques sont disponibles. Ainsi, l'analyse multiple montre que 42% des échantillons sont contaminés par le NIV dans le monde en 2016, à des teneurs d'en moyenne 114 μ g/kg et avec un maximum retrouvé à 2055 μ g/kg, ce qui en fait un contaminant plus présent que la T2 mais moins que le DON.

4.2. Les fumonisines

Le maïs et les produits dérivés du maïs sont, de loin, les aliments les plus contaminés par les FUMs. La présence de FUMs a également été signalée dans les haricots, le riz, le sorgho, les nouilles de blé, le curry ou encore la bière (Ho and Durst, 2003). Bien que les FUMs soient retrouvées un peu partout à travers le monde, leur présence est particulièrement critique dans les cultures de céréales des régions tempérées à chaudes, telles que l'Amérique Centrale, l'Amérique du Sud, le sud de l'Europe, l'Asie et l'Afrique (BIOMIN, 2016).

De manière générale, les FUMs sont les mycotoxines les plus retrouvées dans le monde, comme le montrait l'étude de Rodrigues and Naehrer (2012) dans laquelle 64% des échantillons céréaliers étaient contaminés par des FUMs. En 2016, les taux les plus critiques sont retrouvés en Amérique du Sud (2894 μ g/kg en moyenne, avec un maximum de 171920 μ g/kg retrouvé) et en Asie (2643 μ g/kg en moyenne, avec un maximum de 59709 μ g/kg), où plus de 90% des échantillons de maïs sont contaminés (BIOMIN, 2016).

4.3. La zéaralénone

Cette mycotoxine est essentiellement retrouvée dans le maïs, mais contamine également d'autres céréales telles que l'avoine, l'orge, le blé et le sorgho. Elle est, de plus, très souvent retrouvée en présence de DON dans le blé, l'orge, l'avoine et le maïs, et en présence de FUMs principalement dans le maïs (Yazar and Omurtag, 2008). Chez les animaux, la ZEA étant rapidement biotransformée et excrétée, sa consommation *via* la viande et des produits à base de viande est vraisemblablement peu significative (Creppy, 2002). D'autre part, la ZEA et ses métabolites ainsi que leur forme conjuguée avec l'acide glucuronique peuvent être retrouvés dans le lait de vache, seulement lorsque les bovins ont consommé de très importantes quantités de ZEA (à partir de 544,5 mg/jour pendant 21 jours). Ainsi, il est très peu probable également qu'il y ait un risque lié à la consommation de lait (Prelusky et al., 1990). Enfin, de la ZEA a déjà été détectée dans les œufs de poule (Iqbal et al., 2013).

La principale source d'exposition à la ZEA reste donc la consommation de céréales, particulièrement dans les régions tempérées du monde telles que l'Amérique du Nord, l'Europe et l'Asie, où il s'agit d'une des fusariotoxines les plus répandues (Nesic et al., 2014). L'enquête réalisée par Rodrigues and Naehrer (2012) montrait que 45% des 7049 échantillons céréaliers analysés entre 2009 et 2011 contenaient de la ZEA. En 2016, la dernière enquête de BIOMIN (BIOMIN, 2016) montre que 63% des échantillons céréaliers analysés dans le monde sont contaminés par la ZEA, à des doses pouvant atteindre 6276 μ g/kg ; la moyenne se situant à 64 μ g/kg. De plus, cette même enquête indique que, sur la trentaine d'échantillons de blé, orge et avoine provenant d'Amérique du nord, 32% contiennent de la ZEA avec une teneur moyenne de 4596 μ g/kg et un maximum de 36000 μ g/kg, correspondant aux taux les plus critiques retrouvés dans le monde cette année-là. En revanche, bien que la quantité d'échantillons contaminés à la ZEA soit plus importante en Afrique que dans le reste du monde, les concentrations retrouvées sont les plus faibles (67% du blé contaminé à 45 μ g/kg en moyenne).

4.4. La moniliformine

La MON est couramment retrouvée dans les grains de maïs. Cette mycotoxine est également un contaminant naturel de nombreuses céréales (avoine, orge, blé, seigle, millet, sorgho et riz) provenant de différentes régions du monde, essentiellement en Europe et au Canada (Ioos et al., 2004; Kosiak et al., 2003; Uhlig et al., 2007). De plus, la MON a déjà été retrouvée dans des fruits et légumes (Knaflewski et al., 2008; Sørensen et al., 2009). Par ailleurs, la MON et la FB1 étant produites par les mêmes espèces de champignons (notamment *F. proliferatum*), elles sont très souvent retrouvées simultanément dans les aliments, en particulier dans le maïs (Price et al., 1993). Cette mycotoxine est également retrouvée en présence de DON et d'autres mycotoxines émergentes comme la BEA et les ENNs.

Sur 1378 échantillons céréaliers examinés *via* l'analyse LC-MS/MS multiple (chromatographie liquide couplée à la spectrométrie de masse en tandem) (BIOMIN, 2016), 75% contiennent de la MON, les échantillons de maïs étant les plus concernés (94%). Les teneurs retrouvées dans ces échantillons restent toutefois assez faibles par rapport à d'autres mycotoxines, avec une moyenne de 86 μ g/kg et un maximum de 2560 μ g/kg.

5. Mécanismes d'action et propriétés toxicologiques

5.1. Les trichothécènes

5.1.1. Mécanisme d'action

Les TCTs sont aujourd'hui bien connus comme étant de puissants inhibiteurs de la synthèse protéique chez les eucaryotes. En particulier, il a été montré que la trichodermine inhibiait l'activité de la peptidyltransférase en interférant avec le site actif de cette enzyme sur le ribosome (Stafford and McLaughlin, 1973), tout comme l'ensemble des TCTs qui semblent se lier au même site de liaison ribosomique. En fonction du TCT considéré, ceux-ci peuvent interférer au cours des différentes étapes de la synthèse protéique (initiation, élongation ou encore terminaison). La T2, le DON et le NIV font partie des TCTs de type I, qui inhibent l'étape d'initiation de la synthèse des protéines (Ueno, 1985). De manière générale, le groupement 12-13 époxyde est essentiel à l'inhibition de la synthèse protéique; l'ouverture de ce cycle conduit à la formation de dérivés inactifs. De même, la réduction de la double liaison 9,10 réduit la toxicité de ces composés (Bennett and Klich, 2003; Feinberg and McLaughlin, 1989).

Les TCTs diffèrent dans leur toxicité vis-à-vis des cellules eucaryotes en fonction des caractéristiques chimiques des chaînes latérales (Ueno, 1985). Thompson and Wannemacher Jr. (1986) ont testé dix-neuf mycotoxines 12,13-époxytrichothécènes pour leur capacité relative à inhiber la synthèse des protéines dans les cellules Vero (cellules épithéliales de rein de singe) et les lymphocytes de rat. Ils ont montré un lien étroit entre la structure chimique des TCTs et leur capacité à inhiber la synthèse des protéines. Ainsi, leur étude a montré que la T2 appartenait aux inhibiteurs les plus puissants comparés à DON notamment.

Les autres principaux effets des TCTs sur les cellules eucaryotes sont l'inhibition de la synthèse de l'ADN et de l'ARN, l'inhibition de la fonction mitochondriale, la perturbation de la division cellulaire (incluant l'inhibition de la mitose) ainsi que l'altération de la membrane cellulaire. Dans les cellules animales, les TCTs induisent l'apoptose par la voie intrinsèque mitochondriale et/ou extrinsèque non-mitochondriale et certains déclenchent une réponse au stress ribotoxique, l'activation de protéines kinases activées par des agents mitogènes (MAPKs) ainsi que l'expression de cytokines (Rocha et al., 2005). Cependant, l'inhibition de la synthèse des protéines reste l'effet toxique principal des TCTs. La relation étroite entre la plupart des processus métaboliques cellulaires et la synthèse des protéines suggère que beaucoup des autres effets induits par les TCTs pourraient être des effets secondaires de l'inhibition de la synthèse protéique.

5.1.2. Données toxicologiques

Bien que la sensibilité aux TCTs varie considérablement selon les espèces, ces mycotoxines sont toxiques chez tous les animaux étudiés, la T2 présentant généralement la plus grande toxicité (SCF, 2002). De manière générale, la consommation de TCTs provoque des vomissements, alors qu'un contact cutané peut causer des dermatites (Beasley, 1989; Joffe, 1986). Les autres principaux effets toxiques connus des TCTs chez les animaux sont un retard de croissance, une réduction de la fonction ovarienne associés à des troubles de la reproduction, un refus de s'alimenter et des effets immunosuppresseurs (SCF, 2002).

En particulier, les études menées sur les animaux montrent que la toxine T2 présente des effets immunosuppresseurs et provoque une large gamme d'effets gastro-intestinaux, dermatologiques et neurologiques (Bennett and Klich, 2003; Bouaziz et al., 2013). Trenholm et al. (1989) ont listé et recensé les différentes DL50 (dose létale médiane) et les effets associés sur une large variété d'espèces animales. Ainsi, les espèces les plus sensibles

semblent être les porcs et les rongeurs. Chez le porc, une étude a souligné une réduction du gain de poids corporel causée par la T2 (Weaver et al., 1978) et chez la souris, il a été observé que cette toxine causait des anomalies de la reproduction, et notamment une toxicité embryonnaire ou fœtale (Rousseaux et al., 1986). D'autre part, une étude montrait que les lymphocytes étaient plus sensibles à la toxine T-2 que d'autres types cellulaires (tels que les cellules rénales) (Holladay et al., 1993).

Le DON semble être au contraire un des TCTs les moins toxiques. L'ingestion de DON peut causer une perte de poids et un refus de s'alimenter à faibles doses, alors qu'elle induit des nausées, vomissements et diarrhées à plus fortes doses. De plus, le DON peut significativement altérer l'immunité humorale, l'immunité cellulaire et la résistance de l'hôte chez de nombreux modèles d'expérimentation animale (Cetin and Bullerman, 2005). Par exemple, Tryphonas et al. (1986) ont montré que le DON augmentait la sensibilité de la souris aux infections. Par ailleurs, il a été observé que le DON et le NIV provoquaient un retard de croissance chez la souris (Ohtsubo et al., 1989; Ryu et al., 1988). Toujours chez la souris, le DON cause également des anomalies de la reproduction, telles qu'une augmentation de la mortalité postnatale (Khera et al., 1984).

Différents effets du NIV sur les animaux ont été rapportés dans des études de toxicité aiguë, tels qu'une toxicité de la moelle osseuse, une érythropénie (forme d'anémie) associée à une légère leucopénie (diminution du nombre de lymphocytes dans le sang), une hémorragie et une congestion dans l'intestin ainsi qu'une toxicité pour les organes lymphoïdes (Ryu et al., 1988), mais aussi des diarrhées, des lésions des muqueuses épithéliales de l'intestin, du thymus et des testicules (Ueno, 1984). Les effets toxiques majeurs du NIV observés dans les expériences de toxicité subaiguë, subchronique et chronique chez la souris étaient l'immunotoxicité, l'hématotoxicité et la réduction du gain de poids corporel associé à la réduction de l'apport alimentaire. De plus, le NIV provoque un retard de croissance intra-utérin chez la souris (Ito et al., 1986). Chez le porc, des études de toxicité subaiguë ont montré que le NIV induisait de légers changements pathologiques dans le tractus gastro-intestinal, la rate et les reins ainsi qu'une réduction du gain de poids corporel et de la consommation alimentaire (Pronk et al., 2002). D'autre part, de la même façon que la T2 et le DON, le NIV est un perturbateur du système immunitaire qui inhibe la blastogénèse dans les lymphocytes humains cultivés *in vitro* (Marzocco et al., 2009).

5.1.3. Biotransformation

Après ingestion, il a pu être observé, aussi bien in vivo que in vitro, que la toxine T2 était rapidement métabolisée et éliminée. Différentes réactions de biotransformation (hydrolyse, oxydation, réduction et conjugaison) peuvent se produire au cours du métabolisme de la T2, et une vingtaine de métabolites sont décrits dans la littérature (Li et al., 2011). La plupart de ces substances sont éliminées par excrétion dans les fèces, et de petites quantités de T2 conjuguées à des composés glucoronides seraient également éliminées via l'urine. Les métabolites ainsi que les concentrations retrouvés dans les excréments dépendent fortement de l'espèce étudiée (Yagen and Bialer, 1993). Cependant, la principale voie de biotransformation serait une désacétylation du groupe acétyle C-4 de la toxine T2. Dans les microsomes isolés du foie, du rein et de la rate de divers animaux, la toxine HT2 a d'ailleurs été détectée comme seul métabolite de la toxine T2 (Ohta et al., 1977). Les autres principaux métabolites retrouvés sont le T-2 triol, T-2 tétraol et le néosolaniol. Chez l'humain, il n'existe que quelques études sur le métabolisme de la toxine T2. Johnsen et al. (1988) ont observé la formation de HT2 et de néosolaniol dans les cellules sanguines humaines, produits en quantités égales. Quelques études ont observé une cytotoxicité in vitro plus importante pour la T2 que pour ses métabolites, la HT2 et le néosolaniol, sur différents modèles cellulaires, euxmêmes plus cytotoxiques que le T-2 triol et le T-2 tétraol (Babich and Borenfreund, 1991; Königs et al., 2009).

Le principal métabolite caractérisé de DON est le dé-époxy DON (DOM-1), principalement retrouvé dans l'urine et les fèces des animaux exposés au DON (Yoshizawa et al., 1983), et d'avantage produit par le microbiote de l'intestin ou du rumen plutôt que par le foie (Pestka, 2007). Chez l'humain, Gratz et al. (2013) ont montré que le microbiote fécal était capable de dégrader le DON en DOM-1 (80% et 100% de conversion après 48 et 72h respectivement), alors que Pestka and Smolinski (2005) ont rapporté que les enzymes permettant de métaboliser les substances toxiques et médicaments, tels que les cytochromes de la famille P450 présents dans le foie ne permettaient pas la détoxification du DON en DOM-1 pour protéger l'organisme de la toxicité du DON. Cependant, le foie permet de conjuguer le DON à des composés glucuronides (Gareis et al., 1987; Wu et al., 2007). La récente revue de Maresca (2013) présente les principales voies de biotransformation du DON identifiées chez les espèces mono- et polygastriques.

La principale voie de détoxification des TCTs est la dé-époxydation, qui s'applique également au NIV. La formation du dé-époxy NIV a été mesurée dans des excréments de porcs nourris plusieurs semaines avec des rations contaminées au NIV (Hedman and Pettersson, 1997). Dans ladite étude, presque tout le NIV excrété a été dé-époxydé. Chez le rat, le dé-époxy-NIV est également le principal métabolite excrété après une administration orale répétée (80% de la dose totale retrouvée dans les selles et 1% dans l'urine), alors que le NIV a été détecté à des niveaux beaucoup plus faibles (7% dans les fèces et 1% dans l'urine). Les microorganismes gastro-intestinaux participent probablement à sa dé-époxydation (Onji, 1990).

De manière générale, les bovins sont moins sensibles aux TCTs que la plupart des espèces monogastriques (Beasley, 1989). La capacité du rumen à détoxifier ces toxines *via* une dé-époxydation avant absorption dans le sang explique leur meilleure résistance aux TCTs (Côté et al., 1986).

5.1.4. Valeurs toxicologiques de référence

La dose journalière tolérable (DJT) de la T2 fixée par la JECFA (Joint FAO/WHO Expert Committee on Food Additives) et le SCF (Scientific Commitee of Food) est de 0,06 µg/kg pc/j. Ces comités ont retenu une étude menée chez le porc pendant 3 semaines pour calculer cette DJT (Rafai et al., 1995). Dans cette étude, des effets principalement immunotoxiques et hématotoxiques ont été observés aux faibles doses. Cependant, comme il n'a pas été identifié de dose sans effet néfaste observé (No Observed Adverse Effect Level ou NOAEL), c'est la plus petite dose avec effet (Low Observed Adverse Effect Level ou LOAEL) qui a été retenue, soit 0,029 mg/kg pc/j, à laquelle a été appliqué un facteur de sécurité de 500 pour obtenir la DJT provisoire.

Les DJT des toxines DON et NIV fixées par la JECFA et le SCF ont été établies à partir d'études chroniques menées sur des souris. En particulier, pour le DON, une NOAEL de 100 μ g/kg pc/j avait été établie, avec pour effet toxique pertinent identifié, une diminution de la prise de poids entraînant un retard de croissance (Iverson et al., 1995). Un facteur de sécurité de 100 a ensuite été appliqué pour fixer la DJT du DON à 1 μ g/kg pc/j.

Pour le NIV, aucune des différentes études n'ayant permis d'identifier la NOAEL, c'est la LOAEL qui fut retenue, soit 0,7 mg/kg pc/j. Un facteur de sécurité plus important de 1000 a donc été appliqué pour obtenir la DJT de 0,7 µg/kg pc/j compte tenu du fait que peu de

données sont disponibles sur cette toxine. Les effets toxiques identifiés dans les études de toxicité du NIV correspondaient à des effets immunotoxiques et hématotoxiques.

Le facteur de sécurité (FS) moyen pour déterminer la DJT est de 100 : il permet de prendre en compte les différences interspécifiques (FS=10), afin d'extrapoler les données toxicologiques obtenues sur un modèle animal à l'Homme, ainsi que les différences de sensibilité intraspécifiques (FS=10) au sein de la population humaines (ex : âge, sexe, carences). Ce facteur peut être plus important, comme dans le cas du NIV, s'il s'appuie sur la LOAEL plutôt que sur la NOAEL.

5.2. Les fumonisines

5.2.1. Mécanisme d'action

Les FUMs ont une structure proche de celle de la sphinganine et de la sphingosine, molécules qui constituent le squelette carboné des sphingolipides. Cette ressemblance structurelle suggère que ces mycotoxines perturbent principalement le métabolisme des sphingolipides (et plus particulièrement des céramides, qui correspondent à une sous classe des sphingolipides), impliqués dans de nombreuses fonctions cellulaires telles que l'apoptose, l'inflammation, la croissance et la différenciation cellulaire, la sécrétion protéique ou encore l'endocytose. En particulier, les FUMs inhiberaient la céramide synthase, responsable de l'acylation de la sphinganine et de la sphingosine (Soriano et al., 2005). L'accumulation de la sphinganine dans les tissus serait à l'origine de la potentielle cancérogénicité de la FB1, causant par exemple la synthèse d'ADN non programmée (Schroeder et al., 1994) ou la perturbation du cycle cellulaire (Ramljak et al., 2000). Le ratio sphinganine/sphingosine mesuré dans divers tissus, le sang et l'urine des animaux semble être un indicateur précoce d'une exposition aux FUMs pouvant être utilisé dans le dépistage préclinique des intoxications (Riley et al., 1993; Wang et al., 1991).

5.2.2. Données toxicologiques

En ce qui concerne les effets cliniques majeurs des FUMs, l'œdème pulmonaire porcin (Harrison et al., 1990) et la leucoencéphalomalacie équine (Marasas et al., 1988) sont les principaux effets observés sur la santé animale. Néanmoins, d'autres études *in vivo* ont montré que les FUMs étaient à l'origine, entre autres, d'une insuffisance cardiaque chez les babouins (Kriek et al., 1981), d'une hémorragie cérébrale et de la leucoencéphalomalacie chez les

lapins (Bucci et al., 1996) ou encore d'un cancer du rein chez les rats (Gelderblom et al., 1996; Hard et al., 2001). D'autres effets toxiques ont également été rapportés chez le rat, tels qu'une nécrose du myocarde et un œdème pulmonaire sévère. De façon générale, une toxicité a pu être observée pour toutes les espèces étudiées, notamment dans le foie et les reins qui semblent être des organes cibles de ces mycotoxines. Cependant, la réponse à l'exposition aux FUMs varie en fonction de l'espèce et du sexe de l'animal (Voss et al., 2007). Ainsi, l'espèce animale domestique la plus sensible semble être le cheval, chez lequel la toxicité de la FB1 se manifeste par le développement d'une leucoencéphalomalacie, caractérisée par la présence de lésions nécrotiques liquéfiées dans l'encéphale, et entraînant la mort de l'animal (Marasas et al., 1988).

5.2.3. Biotransformation

En ce qui concerne la biotransformation des FUMs, il existe très peu de preuves que ces mycotoxines soient métabolisées *in vitro* et *in vivo*. Cependant, bien qu'aucune étude ne prouve le métabolisme de ces toxines par les cytochromes P450, la modulation de ces enzymes par les FUMs a pu être observée *in vivo* chez le rat (Spotti et al., 2000) et, plus récemment, chez le poulet (Antonissen et al., 2017). De plus, aucun métabolite hydrolysé n'ayant été retrouvé dans l'urine et dans la bile, on suppose que l'hydrolyse se produirait dans l'intestin sous l'action du microbiote intestinal (Shephard et al., 1994).

5.2.4. Valeurs toxicologiques de référence

La DJT de 2 μ g/kg/p.c./j établie par la JECFA et le SCF pour la FB1, la FB2 et la FB3, seules ou en mélange (SCF, 2003, 2000) a été calculée sur la base de la NOAEL fixée à 0,2 mg /kg pc/j et estimée à partir d'une étude chronique menée sur le rat (effets sur les reins), puis divisée par facteur de sécurité de 100 (JECFA, 2002).

5.3. La zéaralénone

5.3.1. Mécanisme d'action

La ZEA est une mycotoxine œstrogénique non-stéroïdienne pouvant adopter une conformation qui ressemble suffisamment au 17β -œstradiol (principale hormone produite par les ovaires chez la femme) pour lui permettre d'entrer en concurrence avec l'hormone féminine pour se lier aux récepteurs œstrogéniques. En particulier, la ZEA et ses dérivés peuvent se fixer de façon compétitive à deux types de récepteurs des œstrogènes, ER α (ESR1)

et ER β (ESR2), dans les tissus cibles, tels que l'utérus, l'hypothalamus, le foie et les glandes mammaires et pituitaires (Kuiper et al., 1998; Kuiper-Goodman et al., 1987; Takemura et al., 2007; Zinedine et al., 2007). Cependant, le groupement phénol est indispensable à l'activité de la ZEA (Shier, 1998). D'autre part, la ZEA peut augmenter la prolifération des cellules tumorales sensibles à l'æstrogène (Martin et al., 1978). En effet, des adénomes hépatocellulaires et des tumeurs pituitaires ont pu être observés lors d'études de cancérogénicité menées sur du plus long terme chez la souris exposée à de fortes doses (SCF, 2000; Yazar and Omurtag, 2008). De plus, il a été montré que la ZEA induisait la synthèse de créatine kinase, un des événements les plus précoces suivant la fixation des œstrogènes à leurs récepteurs cytoplasmiques (Seeger and Kumar, 1985). Cependant, la fixation aux récepteurs ne conduit pas systématiquement à une réponse œstrogénique. Par exemple, chez des souris en gestation, il a été montré qu'une dose 100 fois plus élevée de ZEA (i.e. 2 µg) était nécessaire pour obtenir le même effet sur le développement des glandes mammaires par rapport au 17 β-œstradiol (i.e. 20 ng) (Hilakivi-Clarke et al., 1998). Après administration orale, la potentialité œstrogénique est également très différente entre les deux molécules, la ZEA étant 650 fois moins efficace que le 17 β -æstradiol (Everett et al., 1987).

Outre son action indirecte sur le métabolisme des hormones stéroïdiennes, la ZEA est également capable d'interférer indirectement dans le métabolisme d'un grand nombre de molécules endogènes et de xénobiotiques. En effet, de nombreuses études indiquent que la ZEA est capable d'activer PXR (Pregnane X Receptor, qui est un récepteur nucléaire) (Ayed-Boussema et al., 2011; Ding et al., 2006; Duca et al., 2012; Fink-Gremmels and Malekinejad, 2007; Mnif et al., 2007). Or, l'expression de gènes impliqués dans le métabolisme de molécules endogènes et de xénobiotiques, tels que le CYP3A4, les glutathion-S-transférases, les sulfotransférases et les UDP-glucuronosyltransferases (UGTs), est régulée par PXR (Ding et al., 2006). Par ailleurs, une diminution de certains marqueurs biochimiques *in vivo* chez le rat et la souris tels que l'aspartate aminotransférase, l'alanine aminotransférase, la phosphatase alcaline, la lactacte déshydrogénase, la créatinine et la bilirubine dans le foie, a pu être observée, suggérant une altération des fonctions hépatiques (Maaroufi et al., 1996; Salah-Abbès et al., 2009).

5.3.2. Données toxicologiques

Chez les animaux, cette fusariotoxine est habituellement non létale et est reconnue pour présenter une toxicité aiguë relativement faible (JECFA, 2000; Zinedine et al., 2007). Son

œstrogénicité se traduit par des modifications fonctionnelles et morphologiques des organes reproducteurs, comme la réduction du tractus génital chez de nombreux animaux de laboratoires (souris, rat, cobayes, hamsters et lapins) ainsi que chez les animaux d'élevage (bovins, ovins, porcins et volailles) (Tatay et al., 2014; Zinedine et al., 2006).

Toutefois, il semblerait que l'espèce la plus sensible soit le cochon, et plus particulièrement la truie. Chez cette espèce, les signes cliniques les plus courants sont, chez le mâle, la diminution des niveaux de sérum de testostérone et de la spermatogenèse ainsi que l'atrophie testiculaire et, chez la femelle, le gonflement de la vulve et du vagin, l'élargissement des glandes mammaires ainsi que d'autres effets sur la reproduction, tels que la baisse de la fertilité, l'augmentation des effets embryocides, la réduction de la taille des portées, la modification du poids des glandes surrénales, de la thyroïde et de l'hypophyse, ainsi qu'un changement des concentrations sériques de progestérone et d'œstradiol. Des effets similaires ont également été observés chez la souris, le rat, le cochon d'Inde, le hamster, le lapin et chez les animaux domestiques. (Creppy, 2002; Nesic et al., 2014; Zinedine et al., 2007). Cependant, aucun effet tératogène n'a pu être observé sur ces différentes espèces (Bacha et al., 1993; JECFA, 2000). Par ailleurs, la ZEA est principalement métabolisée par le foie, qui semble également être une cible importante de cette mycotoxine. En effet, des effets hépatotoxiques ont pu être observés chez le rat et la souris (Maaroufi et al., 1996; Salah-Abbès et al., 2009). D'autre part, des effets hématotoxiques, immunotoxiques et génotoxiques ont pu être montrés suite à l'exposition à la ZEA (Zinedine et al., 2007).

5.3.3. Biotransformation

Les principaux métabolites bioformés de la ZEA sont les α - et β -zéaralénols, également œstrogéniques, qui peuvent eux-mêmes être réduits en α - et β -zéaralanols. La forme α -zéaralénol aurait d'ailleurs un potentiel œstrogénique plus important que la ZEA (Hagler et al., 1979), ce qui serait probablement dû à sa meilleure affinité pour les récepteurs des œstrogènes (Fitzpatrick et al., 1989). Les métabolites de la ZEA ainsi que la ZEA elle-même peuvent être conjugués avec un acide glucuronique (Olsen et al., 1981). La ZEA peut également subir des réactions de sulfoconjugaison, sauf chez le porc, déficitaire en sulfotransférases (Gaumy et al., 2001). Malekinejad et al. (2006) ont, par ailleurs, signalé d'importantes différences dans le métabolisme hépatique de la ZEA chez les animaux. Par exemple, il a été montré que le principal métabolite formé par le foie était le β -zéaralénol chez les bovins et les volailles alors que, chez le cochon, l' α -zéaralénol était la forme

majoritairement formée, ce qui explique que les effets œstrogéniques observés suite à l'ingestion de ZEA sont plus prononcés chez cette espèce. Les cellules intestinales sont également capables de dégrader la ZEA, et chez le cochon, les 4 formes métaboliques sont retrouvées, ainsi que leurs formes conjuguées (JECFA, 2000). Chez l'humain, très peu de données sont disponibles sur la biotransformation de la ZEA. Néanmoins, Videmann et al. (2008) ont montré que la ZEA pouvait être métabolisée par les cellules intestinales humaines principalement sous la forme α -zéaralénol. Mukerjee et al. (2014) ont proposé un schéma synthétique des principales voies de biotransformation de la ZEA chez les mammifères.

5.3.4. Valeurs toxicologiques de référence

La dose temporaire a été fixée par le SCF (SCF, 2000) est de 0,2 μ g/kg p.c./j. Cette valeur a été obtenue en divisant la NOAEL de 40 μ g/kg p.c./j établie à partir d'une étude menée pendant 15 jours sur des truies (effets sur la reproduction) (Edwards et al., 1987b, 1987a) par un facteur de sécurité de 200.

5.4. La moniliformine

5.4.1. Mécanisme d'action

L'action cytotoxique de la MON a été associée à sa capacité à inhiber des enzymes dépendantes de la thiamine pyrophosphatase, dont la pyruvate déshydrogénase, l' α -cétoglutarate déshydrogénase, la pyruvate décarboxylase et l'acétohydroxyacide synthase (Gathercole et al., 1986). Cette inhibition empêcherait alors l'oxydation, entre autres, du pyruvate et de l' α -cétoglutarate, prévenant l'entrée de ces deux métabolites dans le cycle de Krebs et entraînant la réduction de la phosphorylation oxydative (Thiel, 1978). Cela conduirait à une carence en énergie cellulaire, ce qui pourrait expliquer en partie le stress respiratoire, les effets myocardiques et même la mortalité des animaux exposés à la MON (Engelhardt et al., 1989; Gathercole et al., 1986; Kriek et al., 1977). La MON peut également interférer avec le métabolisme des glucides par l'inhibition de la néoglucogenèse et de l'aldose réductase (Deruiter et al., 1993; Pirrung et al., 1996).

Chez les oiseaux, une augmentation significative de la concentration en créatinine sérique et des activités de l'alanine aminotransférase, de l'aspartate aminotransférase et de la créatine kinase a pu être observée suite à une exposition prolongée à la MON, suggérant des lésions hépatiques (Kubena et al., 1999; Reams et al., 1997). Par ailleurs, *in vitro*, à partir des tissus

cardiaques de rat, il a été montré que la MON inhibait certaines enzymes telles que le glutathion peroxydase et le glutathion réductase (enzymes particulièrement importantes dans la protection contre le stress oxydant), suggérant ainsi que le métabolisme des radicaux libres dans le cœur était compromis par l'inhibition de ces enzymes cruciales (Chen et al., 1990). De plus, la cytotoxicité *in vitro* de la MON sur les hépatomes de rats RH, sur les cellules rénales canines MDCK, sur les cellules ovariennes de hamster CHO-K1 (Vesonder et al., 1993) ainsi que sur les lymphocytes humains MIN-GL1 (Visconti et al., 1991) et progéniteurs hématopoïétiques CFU-GM (Ficheux et al., 2012) a également pu être observée. Les effets de cette toxine sont toutefois dépendants du modèle cellulaire testé.

5.4.2. Données toxicologiques

La MON est décrite comme étant fortement toxique, et présente une toxicité comparable à la T2 chez les oiseaux et les rongeurs (Abbas et al., 1990; Allen et al., 1981; Burmeister et al., 1979; Kriek et al., 1977; Nagaraj et al., 1996). D'importants effets cardiotoxiques ont pu être observés, entraînant la mort rapide de ces deux espèces. Chez les rats et les poussins, une faiblesse musculaire progressive, une détresse respiratoire, une dégénérescence du myocarde suivis d'un coma puis de la mort de l'animal ont été observés (Battilani et al., 2009; Kriek et al., 1977). Les oiseaux semblent être parmi les espèces les plus sensibles à la MON, comme le souligne l'étude de Kriek et al. (1977) dans laquelle une DL50 14 fois plus élevée a été obtenue chez le rat que chez le caneton après administration par gavage (respectivement 50 et 3,7 mg/kg). En particulier, chez les oiseaux, les lésions cardiaques se sont avérées être la cause principale de mortalité suite à une alimentation prolongée contenant de la MON. Des études de toxicité chronique ont en effet souligné une cardiotoxicité de la MON chez la dinde à des doses supérieures ou égales à 25 mg/kg d'aliments et une hépatotoxicité à partir de 37 mg/kg. Reams et al. (1997) et Kubena et al. (1999) ont également montré qu'une exposition prolongée des oiseaux à la MON induisait une faible croissance, une augmentation du taux de pyruvate sérique et une cardiopathie, mais aussi un dysfonctionnement hépatique. Dans d'autres études menées sur les volailles, une mortalité élevée a été observée, ainsi que de graves lésions, incluant de l'ascite (qui serait la conséquence de dégâts rénaux et hépatiques), de l'hydropéricardie (résultant de dégâts cardiaques) et des pâleurs du myocarde (Engelhardt et al., 1989). La récente étude de Jonsson et al. (2015) souligne également des effets sur le système immunitaire des rats exposés par voie orale à la MON pendant 28 jours.

5.4.3. Biotransformation

Les données sur l'absorption, la distribution, le métabolisme et l'excrétion de la MON après exposition orale sont très rares. Néanmoins, une récente étude menée chez le rat pendant 28 jours (Jonsson et al., 2015) montre que la MON est rapidement excrétée dans l'urine (entre 20 et 30% quotidiennement) et ne montre pas de signe d'accumulation. De plus, la quantité de MON dans les fèces est inférieure à 2%, suggérant une importante absorption de cette toxine par le tube digestif. Cependant, les possibles métabolites issus de la biotransformation de la MON par l'organisme demeurent encore inconnus.

5.4.4. Valeurs toxicologiques de référence

A ce jour, ni le JECFA, ni le SCF n'ont proposé de dose journalière tolérable pour la MON.

6. Exposition humaine aux fusariotoxines

6.1. Les trichothécènes

6.1.1. Données épidémiologiques

Chez l'Homme, la consommation de produits contaminés par des TCTs serait à l'origine de graves mycotoxicoses. L'un des premiers épisodes d'intoxication pouvant être imputé aux TCTs survint en 1891 en Sibérie Orientale, et fut nommé « maladie chancelante » (Gravier-Rames, 1989). Les affections les plus connues suspectées d'être dues aux TCTs sont l'aleucie toxique alimentaire, plus couramment nommée ATA, décrite en Russie, ainsi que la « moldy corn toxicosis » en Amérique du Nord et la « red mold disease », également appelée « Akakabi byo disease », au Japon. Ces maladies se caractérisent par les mêmes symptômes : diarrhées, vomissements, modifications hématologiques et apparition de pétéchies dans un premier temps, puis intensification de l'altération du système hématopoïétique avec une diminution du nombre de cellules circulantes au cours du troisième stade. En 1940, l'ATA a provoqué le décès de 10% de la population du comté d'Orenburg en Russie. Le mécanisme impliqué dans les troubles hématologiques dus à la forte cytoxicité de la T2 sur les progéniteurs hématopoïétiques a été mise en évidence par la suite. Le Centre International de Recherche sur le Cancer (CIRC ou IARC en anglais) a classé en 1993 les différentes TCTs dans le groupe 3, c'est-à-dire inclassables quant à leur cancérogénicité pour l'Homme, du fait du manque de données chez l'Homme et limitées chez l'animal (WHO-IARC, 1993).

6.1.2. Exposition de la population française

En France, l'EAT1 entreprise par l'ANSES en 2000 pour déterminer les niveaux d'exposition de la population aux TCTs à partir d'aliments « prêts à consommer », montrait que sur les 238 échantillons analysés, seuls 2 et 3 échantillons contenaient respectivement de la HT2 et du NIV (soit environ 1%), et 31 contenaient du DON (soit 13%) (Leblanc et al., 2005). Cette étude soulignait également qu'aucun des individus adultes (15 ans et plus) et enfants (de 3 à 14 ans) ne dépassaient la limite maximale recommandée par le JECFA et le SCF pour le NIV (fixée à 0,7 µg/kg p.c./j), et que 0,4% des adultes et 4 % des enfants étaient susceptibles de dépasser la limite pour le DON (fixée à 1 µg/kg p.c./j). En ce qui concerne la population végétarienne, environ 4% étaient susceptibles de dépasser la limite fixée pour le NIV et entre 4 et 5% pour le DON selon le type de régime alimentaire (végétalien, ovolactovégétarien ou lactovégétarien) (AFSSA, 2009; Leblanc et al., 2005). L'exposition de la population estimée lors de l'EAT2 réalisée entre 2006 et 2009 semble moins importante que celle estimée lors de l'EAT1 pour le NIV, alors que celle au DON augmente (Sirot et al., 2013). De plus, l'EAT2 souligne que le risque peut être écarté pour la population générale concernant le NIV, mais pas concernant le DON et ses dérivés acétylés pour lesquels les calculs d'exposition montrent des dépassements des valeurs toxicologiques de référence.

6.2. Les fumonisines

6.2.1. Données épidémiologiques

Chez l'Homme, des études épidémiologiques semblent établir un lien entre le cancer de l'œsophage et l'ingestion de maïs contaminé par *Fusarium verticillioides* (Albertini et al., 2000). Plus particulièrement, la présence de FB1 dans les céréales a été associée à l'incidence d'un taux élevé de cancers de l'œsophage en Afrique du sud (Gelderblom et al., 1992; Rheeder et al., 1992), dans le nord de l'Italie (Franceschi et al., 1990), en Chine (Chu and Li, 1994; Yoshizawa et al., 1994) et en Iran (Shephard et al., 2000), ainsi que pour des cancers du foie au stade primaire observés dans certaines zones endémiques de la République populaire de Chine (Ueno et al., 1997). Par ailleurs, certaines études suggèrent que les FUMs peuvent augmenter le risque d'anomalies du tube neural chez les populations qui consomment de grandes quantités de maïs contaminé (Hendricks, 1999; Missmer et al., 2006). Le risque cancérigène pour l'Homme lié à la consommation de FUMs a été évalué par le CIRC à un niveau 2B (probablement cancérigène) (WHO-IARC, 2002). Malgré l'existence de ces

preuves épidémiologiques, les mécanismes liés aux effets carcinogènes de la FB1 demeurent encore incertains.

6.2.2. Exposition de la population française

En France, l'ETA1 entreprise en 2000 (Leblanc et al., 2005) sur le niveau de consommation et d'exposition aux FUMs pour l'ensemble de la population française (adultes, enfants, végétariens) indiquait que l'apport théorique était inférieur à la DJT établie par le JECFA et le SCF (fixée à 2 μ g/kg/p.c./j). L'exposition de la population estimée lors de l'EAT2 de 2006 semble équivalente à celle estimée au cours de l'EAT1 pour ces toxines (Sirot et al., 2013).

6.3. La zéaralénone

6.3.1. Données épidémiologiques

Chez l'Homme, la ZEA a été suspectée d'être l'agent responsable d'une épidémie de thélarches prématurées (développement prématuré des seins) chez de jeunes enfants à Porto-Rico entre les années 1978 et 1981 (Sáenz de Rodríguez, 1984; Sáenz de Rodriguez et al., 1985). D'autres cas de pubertés précoces ont également été signalés dans la région du sud-est de la Hongrie, où de la ZEA avait été retrouvée à de fortes concentrations dans le sérum des patients et également dans des échantillons d'excédents de nourriture collectés chez ces mêmes patients (Szuets et al., 1997). D'autres études plus récentes ont démontré le potentiel de la ZEA à stimuler la croissance de cellules cancéreuses dans le sein (Ahamed et al., 2001; Yu et al., 2005). Le risque cancérigène pour l'Homme lié à la consommation de ZEA a été évalué par le CIRC à un niveau 3 (c'est-à-dire inclassable quant à sa cancérogénicité pour l'Homme) (WHO-IARC, 1993).

6.3.2. Exposition de la population française

L'étude de Leblanc et al. (2005) entreprise en France en 2000 (dans le cadre de l'EAT1) a souligné que la proportion d'individus dont l'apport théorique de ZEA dépassait la DJT temporaire (fixée à de $0,2 \mu g/kg p.c./j.$) était de 2,5% pour les enfants de 3 à 14 ans et de 31% pour les végétaliens. L'exposition de la population estimée lors de l'EAT1 diminue au cours de l'EAT2 pour cette mycotoxine, et le risque asocié à sa présence dans l'alimentation peut être écarté pour la population générale (Sirot et al., 2013).

6.4. La moniliformine

6.4.1. Données épidémiologiques

Aucun cas de mycotoxicose associé à l'ingestion de MON n'a été rapporté mais une étude *in vivo* menée chez de jeunes rats et des cannetons a mis en évidence que la MON augmentait la perméabilité cardiaque, suggérant un mécanisme qui pourrait induire la maladie de Keshan (cardiomyopathie) chez l'Homme (Zhang and Li, 1989). Cette maladie sévie dans diverses régions de Chine où le maïs est contaminé par des *Fusarium* producteurs de MON (Yu et al., 1995). Les personnes atteintes de cette affection souffrent de problèmes cardiaques semblables à ceux observés lors d'études expérimentales (Wu et al., 1995).

6.4.2. Exposition de la population française

Aucune étude n'a encore étudié l'apport théorique de la MON via l'alimentation en France.

Partie III : Présentation du projet de thèse

Le manque de prise en compte réglementaire des multi-contaminations aux mycotoxines des céréales et produits céréaliers est principalement dû à la rareté des données toxicologiques ; de plus, les effets des combinaisons de mycotoxines sur les mécanismes cellulaires sont insuffisamment connus. Dans ce contexte, l'objectif général de ce projet de thèse était de mieux caractériser l'incidence de l'exposition combinée aux mycotoxines sur les cellules humaines dans des conditions d'exposition aiguës (exposition unique à de fortes doses) et chroniques (expositions répétées à de plus faibles doses) afin d'obtenir une meilleure appréciation du risque « mycotoxines » dans les céréales. Plus précisément, il s'agissait d'étudier l'effet cytotoxique des combinaisons de fusariotoxines ainsi que d'identifier les mécanismes cellulaires impliqués dans la toxicité de ces mélanges *via* des approches toxicologiques et protéomiques en utilisant différentes lignées cellulaires humaines (inestinales, monocytaires et hépatocytaires) représentatives des barrières de défense de l'organe de détoxification, cibles privilégiées des mycotoxines. Mon travail de thèse s'est alors articulé en 4 parties :

- (i) Tout d'abord, l'effet immuno-toxique de six fusariotoxines (DON, NIV, T2, FB1, ZEA et MON) et de quatre mélanges binaires de fusariotoxines (DON+MON, DON+FB1, DON+ZEA et NIV+T2) a été évalué *via* l'utilisation de la lignée monocytaire humaine THP-1 dans des conditions d'expositions aiguës. Plus particulièrement, les effets de ces mycotoxines ont été étudiés sur la viabilité et les mécanismes de mortalité cellulaire, sur l'expression de certains marqueurs de surface ainsi que sur les principales voies de signalisation impliquées dans la croissance, le développement et la différenciation cellulaire. En effet, l'état fonctionnel des cellules dans ce type d'étude n'est pas toujours étudié et pourrait pourtant permettre une meilleure compréhension des phénomènes associés à la cytotoxicité ainsi que de mieux appréhender la réponse cellulaire face à une exposition aux mycotoxines.
- (ii) Dans un second temps, ces mêmes mycotoxines et mélanges ont été utilisés pour évaluer, cette fois, leur pouvoir hépatotoxique sur la lignée hépatocytaire humaine HepaRG, un modèle cellulaire particulièrement pertinent, toujours dans des conditions d'expositions aiguës. De plus, l'effet du mélange DON+ZEA, un des plus répandus dans les céréales, a été évalué à des concentrations cytotoxiques très faibles sur le protéome global des cellules hépatiques HepaRG proliférantes *via* une analyse LC-

MS/MS afin d'identifier de potentiels changements de l'état fonctionnel des cellules non suspectés par les analyses toxicologiques. En effet, très peu de données sont aujourd'hui disponibles sur les mécanismes de réponse cellulaire à l'exposition aux mycotoxines, notamment en raison du manque d'analyses non ciblées à l'échelle moléculaire.

- (iii) Dans une troisième partie, afin de comparer les effets hépatotoxiques observés après une exposition chronique par rapport aux résultats obtenus en conditions aiguës, nous nous sommes intéressés aux effets toxicologiques induits par le DON et la ZEA à des doses réglementaires, seuls et en mélange, après 2, 4 et 6 semaines d'exposition. En raison de la difficulté à maintenir des cultures cellulaires *in vitro* à confluence pendant plusieurs semaines tout en conservant une activité métabolique stable, les données sur la toxicité chronique des mycotoxines, aussi bien individuellement qu'en mélange, sont quasi-inexistantes. Cette étude apparaît donc particulièrement d'intérêt pour une meilleure caractérisation du risque « mycotoxines » pour le consommateur, exposé parfois tout au long de sa vie à ces contaminants *via* son alimentation.
- (iv) Enfin, afin de développer un modèle *in vitro* intégrant l'ensemble des lignées cellulaires étudiées et pour mieux prendre en compte les interactions inter-cellulaires existantes *in vivo*, la toxicité individuelle et combinée du DON et de la ZEA dans des conditions d'exposition aiguës a été testée *via* l'utilisation de modèles en coculture faisant intervenir les cellules Caco-2, les THP-1 et les HepaRG, toujours dans un état non différencié. Dans un souci éthique visant à limiter les expérimentations animales, il est en effet important de développer des modèles *in vitro* plus proches de conditions *in vivo*. Or, aujourd'hui encore, la grande majorité des études toxicologiques *in vitro* concernant les mycotoxines sont réalisées sur des cultures cellulaires classiques ne faisant intervenir qu'un seul type cellulaire à la fois.

1. Choix des toxines et mélanges

Pour notre étude, six fusariotoxines ont été sélectionnées, dont cinq « majeures », le DON, le NIV, la T2, la FB1 et la ZEA, et une mycotoxine « émergente », la MON. Le choix des mycotoxines a été établi sur la base de leur occurrence dans les produits céréaliers européens et/ou de leur importante toxicité sur la santé humaine et animale. De plus, parmi l'infinité de mélanges de mycotoxines retrouvés dans l'alimentation, nous avons choisi les quatre

mélanges binaires suivants : DON+MON, DON+FB1, DON+ZEA et NIV+T2. De manière générale, les mélanges ont été sélectionnés sur la base d'associations de mycotoxines produites par les mêmes espèces de *Fusarium* et donc couramment retrouvées dans les produits céréaliers, comme rapporté dans la *review* publiée. Les principales espèces de *Fusarium* productrices de ces mycotoxines ont été reportées dans le tableau 4.

Le DON est la mycotoxine la plus rencontrée dans nos régions, avec plus de 70% des échantillons céréaliers européens contaminés en 2016, le maïs étant particulièrement touché par cette mycotoxine. De même, la MON est l'une des mycotoxines émergentes les plus retrouvées dans les céréales, en particulier dans le maïs pour lequel 94% des échantillons analysés en 2016 étaient contaminés (BIOMIN, 2016). Ces deux mycotoxines sont souvent retrouvées ensemble dans les céréales, essentiellement dans le maïs, ce qui s'explique par la capacité de nombreuses espèces de champignon, à savoir F. acuminatum, F. avenaceum, F. culmorum, F. oxysporum, F. sporotrichioides ou encore F. tricinctum, à produire ces deux mycotoxines (tableau 4). De plus, aucune étude in vitro ne s'est encore intéressée aux effets toxicologiques combinés de ces deux mycotoxines. D'autre part, la FB1 est également, avec le DON, une des mycotoxines majeures les plus retrouvées dans le maïs (environ 70% du maïs contaminé en Europe et plus de 90% en Afrique, Asie et Amérique du Sud (BIOMIN, 2016)), ce qui en fait un mélange fréquemment rencontré dans cette matrice et encore peu étudié. De plus, F. oxysporum est capable de sécréter ces deux mycotoxines (tableau 4). En ce qui concerne le mélange DON+ZEA, il s'agit de la combinaison la plus retrouvée dans les céréales, notamment en Europe et en Amérique du Nord (Smith et al., 2016), ce qui s'explique en partie par la capacité de nombreuses espèces de champignons à produire ces deux mycotoxines, telles que F. cerealis, F. culmorum, F. graminearum, F. pseudograminearum ou encore F. sporotrichioides (tableau 4). Enfin, les deux TCTs, NIV et T2, sont également souvent produits par les mêmes champignons, à savoir F. culmorum, F. kyushuense et F. poae (tableau 4). De plus, bien que la T2 soit l'une des fusariotoxines les plus toxiques et que la NIV soit très présente dans les céréales (plus de 40% des échantillons céréaliers contaminés dans le monde en 2016 (BIOMIN, 2016)), elles ne sont pas encore réglementées par l'Union Européenne, et peu de données sur leurs effets toxicologiques combinés sont disponibles.
Tableau 4 : Quelques espèces de *Fusarium* produisant les mycotoxines DON, NIV, T2, FB1, ZEA et MON ; *les croix en gras correspondent aux espèces productrices majoritaires.* (Données synthétisées à partir des références bibliographiques suivantes : Bennett and Klich, 2003; Bezerra da Rocha et al., 2014; Bosco and Molle, 2012; Bryden, 2012; D'Mello and Macdonald, 1997; Doohan et al., 2003; Eriksen, 1998; Glenn, 2007; Jestoi, 2008; Marin et al., 2013; Nesic et al., 2014; Smith et al., 2016; Thrane et al., 2004; Ueno, 1980; Yazar and Omurtag, 2008).

	DON	NIV	T2	FB1	ZEA	MON
F. acuminatum	Х		Х			Х
F. anthophilum				Х		
F. avenaceum	Х					Х
F. cerealis	Х	Х			Х	
F. culmorum	X	Х	Х		Х	Х
F. dlamini				Х		
F. equiseti			Х		Х	Х
F. fujikuroi				Х		Х
F. globosum				Х		
F. graminearum	X	Х			X	
F. incarnatum			Х		Х	
F. kyushuense		Х	Х			
F. langsethiae			Х			
F. napiforme				Х		Х
F. nygamai				Х		Х
F. oxysporum	Х			Х		Х
F. poae		Х	Х			
F. polyphialidicum				Х		
F. proliferatum				Х		X
F. pseudograminearum	Х				Х	
F. pseudonygamai				Х		Х
F. solani	Х		Х			
F. sporotrichioides	Х		Х		Х	Х
F. subglutinans				Х		Х
F. thapsinum				Х		Х
F. tricinctum	Х		X			X
F. verticillioides				Χ	Х	Х

2. Choix des modèles cellulaires

Les modèles d'étude in vitro sont aujourd'hui de plus en plus utilisés car ils répondent aux recommandations de la législation en vigueur en Europe et en Amérique du Nord, visant à limiter l'utilisation d'animaux pour les expériences scientifiques. En particulier, ces législations encouragent la règle des « 3R » (Réduire, Raffiner, Remplacer) élaborée en 1959, qui constitue le fondement de la démarche éthique appliquée à l'expérimentation animale. De plus, les modèles in vitro sont développés en milieu contrôlé, et leur utilisation permet la mise en évidence de nombreux mécanismes cellulaires grâce, par exemple, au développement d'outils de marquage intracellulaires. D'autre part, l'emploi de lignées cellulaires in vitro (cellules transformées/immortalisées) comporte certains avantages par rapport à celui de cellules primaires car, non seulement il permet de s'affranchir des contraintes liées aux problèmes d'éthique, à l'accessibilité et à la disponibilité des donneurs, mais il permet aussi de minimiser les effets dus aux variations phénotypiques et génétiques inter-donneurs, et de réduire les problèmes liés à la durée de vie limitée des modèles primaires. Dans ce projet de thèse, face aux manques de données toxicologiques sur l'humain, trois lignées cellulaires humaines ont été choisies pour l'étude des effets aiguës et chroniques de fusariotoxines d'intérêt, seules et en mélange : les cellules de l'épithélium intestinal Caco-2, les monocytes THP-1 et les cellules d'origine hépatique HepaRG. En effet, l'épithélium intestinal constitue la première barrière de défense de l'hôte suite à l'ingestion de contaminants alimentaires, tels que les mycotoxines. Cette barrière est constituée d'une monocouche d'enterocytes matures, régulièrement renouvelée (tous les 4 à 6 jours chez l'Homme), grâce à la présence de nombreuses cellules immatures présentes dans des cryptes de la paroi intestinale. Ces cellules indifférenciées sont donc également sujettes à une exposition aux mycotoxines après ingestion. Par la suite, les mycotoxines qui franchissent la barrière intestinale, se retrouvent dans le sang. Or, les monocytes, qui sont des cellules du sytème immunitaire, sont présents dans le sang et pouvent se différencier en macrophages dans les tissus. Ensuite, les mycotoxines vont se retrouver, en partie, dans le foie, organe de détoxification de notre organisme. C'est pourquoi, dans ce projet de thèse, pour une meilleure comparaison des modèles, toutes les lignées cellulaires sélectionnées ont été étudiées dans un état non différencié. Il est à noter que la différienciation des cellules a un impact sur leur sensibilité aux mycotoxines, et aux xénobiotiques en général, puisque les fonctions cellulaires évoluent au cours de la différenciation.

2.1. La lignée intestinale humaine Caco-2

La barrière intestinale joue un rôle important dans la protection de l'organisme contre les substances toxiques ingérées. Elle représente également un des sites majeurs d'exposition aux agents potentiellement toxiques en raison de sa vaste zone d'exposition et de son rôle physiologique dans le transfert de nutriments de la lumière vers le sang. Le tractus gastrointestinal, en plus de son rôle dans l'absorption des xénobiotiques, participe également activement à leur biotransformation. Certaines mycotoxines, comme le DON, sont d'ailleurs essentiellement dégradées aux niveaux du tube digestif plutôt que par le foie (Pestka, 2007).

La lignée cellulaire tumorale humaine Caco-2 d'origine intestinale, obtenue à partir d'un adénocarcinome colique humain, est très couramment utilisée dans les études de toxicologie in vitro en tant que modèle pour mimer la barrière intestinale. Bien que des problèmes de reproductibilité aient pu être rapportés dans la littérature, rendant difficile la comparaison des résultats entre laboratoires, cette lignée reste largement utilisée depuis une trentaine d'années dans les études in vitro. Ces problèmes ont notamment été attribués à la variabilité intrinsèque des cellules Caco-2 utilisées dans les différents laboratoires ainsi qu'aux différentes conditions liées à la culture, telles que le type de sérum animal utilisé, les suppléments ajoutés au milieu de culture, le nombre de passage et la source des clones (Sambuy et al., 2005; Zucco et al., 2005). Des protocoles de culture optimisés ont depuis été proposés dans un souci de standardisation (Natoli et al., 2012). Par ailleurs, ces cellules ont la particularité de pouvoir se différencier spontanément, ce qui conduit alors à la formation d'une monocouche de cellules intestinales polarisées exprimant plusieurs caractéristiques morphologiques et fonctionnelles d'entérocytes intestinaux matures (Sambuy et al., 2005). De plus, différentes études ont souligné que ces cellules présentaient une meilleure différenciation entérocytaire morphologique et fonctionnelle que la plupart des autres lignées cellulaires issues du carcinome du côlon (Chantret et al., 1988; Sambuy et al., 2005). Bien que les cellules Caco-2 proviennent du carcinome du côlon humain, elles acquièrent en cours de culture certaines caractéristiques des cellules absorbantes de l'intestin (présence de microvillosités, de transporteurs spécifiques (des sucres, des acides aminés ou encore de divers médicaments) et d'enzymes du processus de métabolisation du type hydrolases, mimant alors une barrière intestinale fonctionnelle (Blais et al., 1987; Chantret et al., 1988; Dantzig and Bergin, 1990; Hidalgo et al., 1989; Hidalgo and Borchardt, 1990a, 1990b; Hilgers et al., 1990; Yamashita et al., 2000). De plus, l'adhésion des cellules via des jonctions serrées formées du côté apical de la monocouche cellulaire permet deux types de transports des médicaments à travers la monocouche épithéliale : le transport transcellulaire (via la cellule par transport passif, actif ou encore endocytose) et le passage paracellulaire (via les jonctions serrées) (Tanaka et al., 1995; Maubon et al., 2007). Dans le but de reproduire au mieux les conditions stériques in vivo de l'intestin, les cellules Caco-2 sont généralement cultivées sur des membranes perméables permettant l'accès libre des ions et des nutriments des deux côtés de la monocouche cellulaire (côté apical, correspondant à la lumière intestinale, et côté basal, représentant le milieu intérieur). C'est d'ailleurs dans de telles conditions de culture qu'une amélioration de la différenciation entérocytaire de ces cellules a pu être obtenue, et que les cellules Caco-2 sont aujourd'hui utilisées comme modèle physiologique pour le transport intestinal et les études de toxicité (Artursson, 1990; Hidalgo et al., 1989; Hilgers et al., 1990; Wilson et al., 1990). En effet, ce modèle est connu pour mimer la membrane intestinale in vivo, et permet ainsi d'investiguer la perméabilité intestinale d'un composé chimique, c'est-àdire sa capacité à traverser la paroi intestinale pour rejoindre la circulation sanguine et se distribuer dans l'organisme. Plusieurs études ont déjà montré la bonne corrélation entre la perméabilité apparente in vitro des monocouches Caco-2 de certains composés d'intérêts et l'absorption orale chez l'humain de ces composés (Artursson and Karlsson, 1991; Cheng et al., 2008; Lennernäs et al., 1996; Rubas et al., 1993; Stewart et al., 1995; Tanaka et al., 1995; Yamashita et al., 1997, 2000). En particulier, les tests les plus couramment utilisés pour surveiller l'intégrité de la couche cellulaire sont la mesure de la résistance électrique transépithéliale (TEER) lorsque les cellules sont à confluence et la mesure de la perméabilité via l'utilisation de marqueurs moléculaires.

2.2. La lignée monocytaire humaine THP-1

L'épithélium intestinal et le système immunitaire constituent les premières barrières de défense de l'hôte suite à l'ingestion de mycotoxines. Le système immunitaire est d'ailleurs très sensible aux mycotoxines (Corrier, 1991; Wu et al., 2014). Cependant, bien que la plupart des fusariotoxines soient de puissants immuno-modulateurs qui perturbent, entre autres, la différenciation des monocytes humains en macrophages ou cellules dendritiques (Ficheux et al., 2013; Hymery et al., 2009; Solhaug et al., 2016), peu de données sont disponibles sur les effets de l'exposition et de la co-exposition aux mycotoxines sur le système immunitaire. La fonction des monocytes, notamment leur différenciation en macrophages, est d'ailleurs une fonction centrale de la réponse immunitaire. En effet, les monocytes et les macrophages ont un rôle de i) reconnaissance des pathogènes étrangers (tels que les bactéries, les champignons,

les virus) ansi que des toxines ou encore des polluants *via* les différents types de récepteurs à reconnaissance de motifs (PRRs) présents sur leur surface, ii) prolifération pour augmenter le nombre de cellules capables d'éliminer ces pathogènes, iii) production de cytokines et de chimiokines pro-inflammatoires permettant l'intervention de cellules effectrices sur le site d'infection par exemple, ainsi que des cytokines anti-inflammatoires lorsque l'infection est sous contrôle et iv) phagocytose pour englober et digérer ces pathogènes (Chanput et al., 2014; Si-Tahar et al., 2009).

La lignée monocytaire THP-1 est une lignée aujourd'hui largement utilisée, aussi bien à l'état de monocyte que de macrophage, pour l'étude des fonctions, des mécanismes, des voies de signalisation et du transport de nutriments et de médicaments chez ces deux types cellulaires (Chanput et al., 2014). Cette lignée a été isolée en 1980 à partir du sang périphérique d'un enfant de 1 an souffrant de leucémie monocytaire aiguë (Tsuchiya et al., 1980). De nombreuses études ont montré que les cellules THP-1 avaient des propriétés morphologiques et fonctionnelles proches des monocytes et des macrophages humains primaires (PBMC), tels que l'expression de marqueurs caractéristiques de monocytes immatures en suspension et de marqueurs de différenciation (Hjort et al., 2003; Kramer and Wray, 2002; Sakamoto et al., 2001; Tsuchiya et al., 1982; Ueki et al., 2002). De plus, cette lignée semble être un modèle cellulaire in vitro pertinent pour étudier l'immunotoxicité des mycotoxines (Fontaine et al., 2015). D'autre part, les THP-1 représentent un modèle d'étude intéressant, notamment pour explorer la réponse inflammatoire provoquée par ces médicaments et caractérisée par la libération de cytokines et de chimiokines pro-inflammatoires (Mizuno et al., 2011). Par ailleurs, des études ont montré que les THP-1 étaient capables de métaboliser certains composés hépatotoxiques (mébendazole, ximélagatran, terbinafine, troglitazone) (Edling et al., 2008, 2009; Mizuno et al., 2011). Cependant, ces cellules ne sont pas les plus appropriées pour étudier la biotransformation des médicaments, principalement métabolisés dans le foie par des hépatocytes.

2.3. La lignée hépatocytaire humaine HepaRG

Le foie étant l'organe de détoxification de référence de l'organisme, les cellules hépatocytaires représentent un des modèles d'étude *in vitro* les plus adaptés à l'étude du métabolisme des xénobiotiques et aux études toxicologiques. En effet, les fonctions du foie sont principalement assurées par les hépatocytes qui représentent environ 80% de la population cellulaire totale. Par ailleurs, l'altération des fonctions hépatiques est l'un des

principaux effets observés sur les animaux suite à l'exposition à de fortes doses de mycotoxines (Pitt, 2000). Cependant, plus de 50% des médicaments induisant des lésions hépatiques dans les essais cliniques humains ne sont pas hépatotoxiques chez les animaux, ce qui souligne l'importance d'utiliser des hépatocytes humains pour évaluer de façon plus précise la toxicité d'un médicament, ou d'autres molécules bioactives, *in vitro* chez l'Homme (Olson et al., 2000). Néanmoins, comme pour l'ensemble des cellules primaires, les hépatocytes humains primaires sont phénotypiquement instables, présentent une durée de vie limitée et une grande variabilité inter-donneurs. D'un autre côté, les lignées hépatocytaires d'origine tumorale ou obtenues par immortalisation oncogénique, telles que les cellules HepG2 et C3A, sont dépourvues de certaines fonctions importantes spécifiques du foie. En particulier, ces lignées ne possèdent pas certains cytochromes majeurs de la famille P450, impliqués dans le métabolisme des xénobiotiques, et présentent donc un intérêt limité pour des études pharmaceutiques et thérapeutiques (Guguen-Guillouzo and Guillouzo, 2010; Wu et al., 2016).

En revanche, la lignée hépatocytaire humaine HepaRG, obtenue à partir d'une tumeur du foie d'une patiente souffrant d'hépatocarcinome et d'une infection à l'hépatite C (Gripon et al., 2002), semble être un bon substitut aux hépatocytes primaires pour les études de toxicologie. En effet, cette lignée possède à la fois les performances métaboliques des hépatocytes humains primaires et les capacités de croissance des lignées hépatocytaires (Guillouzo et al., 2007). Plus particulièrement, les cellules HepaRG expriment de nombreuses fonctions spécifiques du foie telles que les principaux cytochromes P450 et certains récepteurs nucléaires à des niveaux comparables à ceux trouvés dans les hépatocytes humains primaires. Les HepaRG expriment également de nombreuses autres fonctions telles que des enzymes de la Phase II du métabolisme des xénobiotiques, des transporteurs membranaires, de l'albumine, de l'haptoglobine ou encore de l'aldolase B. De plus, ces cellules peuvent être maintenues à confluence pendant plusieurs semaines tout en conservant une activité métabolique stable, ce qui en fait le modèle idéal pour l'étude des paramètres du métabolisme des médicaments ainsi que des effets aigus et chroniques des xénobiotiques sur le foie humain (Aninat et al., 2006; Anthérieu et al., 2012; Guillouzo et al., 2007; Jossé et al., 2008). D'autre part, les cellules HepaRG possèdent des caractéristiques uniques : lorsqu'elles sont détachées et réensemencées à faible densité, elles sont capables de se dédifférencier en réacquérant une morphologie indifférenciée allongée et en se divisant de façon très active pour arriver à confluence rapidement. Suite à cela, deux types de populations cellulaires

morphologiquement différentes peuvent être distinguées : des colonies formées de grappes de cellules épithéliales granulaires ressemblant à des hépatocytes, entourées de cellules plus aplaties. Ces cellules peuvent être différenciées respectivement (avec ajout de DMSO dans le milieu de culture) en cellules plus granulaires possédant un ou deux noyaux et ressemblant étroitement à des hépatocytes primaires adultes pour les premières, et en cellules épithéliales biliaires pour les secondes. Les cellules de type hépatocytaire représentent environ 50 à 55% de la population cellulaire totale (Cerec et al., 2007). Les cellules allongées indifférenciées (hépatoblastes) expriment des marqueurs de cellules progénitrices du foie tandis que les deux types de cellules identifiés à confluence expriment des marqueurs d'hépatocytes et de cellules épithéliales biliaires, respectivement. Les deux états cellulaires (indifférencié et différencié) issus de cette lignée peuvent être utilisés et comparés dans le cadre d'études toxicologiques (Guillouzo et al., 2007). Par ailleurs, une étude récente a montré que, parmi différents modèles hépatocytaires humains (L-02, HepG2, HepaRG et hiHeps), les cellules HepaRG étaient les plus adaptées pour évaluer le potentiel d'un médicament à induire des lésions hépatiques (sur plus de 17 médicaments testés) (Wu et al., 2016). Les auteurs suggèrent même que la lignée cellulaire HepaRG pourrait être utilisée dans le dépistage des médicaments induisant des lésions hépatiques pour une extrapolation in vitro à in vivo. Une étude plus récente encore a utilisé les HepaRG pour le développement et l'application d'un modèle PDB (Physiologically Based Dynamic) permettant l'extrapolation in vitro à in vivo (Paini et al., 2017). Dans le cadre de l'investigation des effets hépatotoxiques in vitro individuels et/ou combinés des mycotoxines, seules quelques études ont utilisé ce modèle, essentiellement pour l'aflatoxine B1 (Jossé et al., 2008; Mueller et al., 2014). Les HepaRG représentent donc un modèle innovant pour ce type d'étude toxicologique.

3. Choix des approches mécanistiques

La mortalité cellulaire est un critère d'évaluation de la toxicité d'un xénobiotique couramment étudié. Bien que cette mesure soit nécessaire pour évaluer la cytotoxicité des mycotoxines, elle demeure insuffisante pour juger l'état fonctionnel des cellules et appréhender la réponse cellulaire à l'exposition à un xénobiotique considéré. En effet, les perturbations des équilibres homéostatiques, en particulier dans le cadre d'expositions chroniques, peuvent entraîner des modifications plus subtiles, dont le résultat pourrait apparaître plus tardivement. C'est pourquoi, dans le cas des cellules hépatiques, l'étude de l'expression de certaines enzymes impliquées dans la détoxification des xénobiotoques, ainsi que d'autres facteurs spécifiques du foie (tels que l'albumine, la transferine et l'aldolase B par exemple), pourrait apporter des informations importantes sur l'état fonctionnel de ces cellules, aussi bien sur des expositions à court terme, qu'à plus long terme.

D'autre part, face au manque de données concernant les mécanismes cellulaires de réponse aux dommages causés par l'exposition et à la co-exposition aux mycotoxines, il devient urgent d'identifier les processus biologiques affectés afin de mieux définir les conséquences de ces expositions sur le plan toxicologique. Certaines voies de signalisation intracellulaires ont déjà pu être identifiées comme étant d'importants médiateurs impliqués dans la transduction des signaux du stress. En particulier, les MAPKs constituent une famille de protéines kinases classées en 3 sous-familles, les extra-cellular signal-regulated (ERK) 1/2, les c-Jun N-terminal kinases (JNKs), et p38 MAPK, qui régulent de façon coordonnée de nombreux processus cellulaires clés tels que l'induction des gènes, l'apoptose, la prolifération, la différenciation, le stress cellulaire et la réponse inflammatoire (Johnson and Lapadat, 2002). Les MAPKs pourraient donc représenter des cibles intéressantes pour l'étude des effets des mycotoxines sur les cellules. De plus, il a pu être montré que les MAPKs pouvaient être activées quelques minutes après l'induction au stress. Leur activation pourrait alors renseigner sur les perturbations de l'organisme à un stade précoce, d'où leur intérêt en toxicologie.

Ainsi, l'étude ciblée de l'expression de certains gènes ou protéines intracellulaires apparaît complémentaire à l'évaluation de la cytotoxicité des mycotoxines. Néanmoins, dans le but d'identifier ou de mettre en évidence de nouveaux biomarqueurs d'exposition ou de générer de nouvelles connaissances sur le plan mécanistique, les approches non ciblées, du type « omique » semblent particulièrement adaptées. Il s'agit de techniques d'analyse à haut débit qui permettent la mesure simultanée d'un grand nombre de paramètres associés au fonctionnement, et donc également au dysfonctionnement, d'une cellule dans le cadre d'études *in vitro*. Ces méthodes comprennent principalement la transcriptomique (analyse fonctionnelle des ARN messagers transcrits), la protéomique (analyse de l'ensemble des protéines identifiées à partir d'un génome, de leur localisation, leur structure, leurs fonctions, leurs interactions) et la métabolomique (étude de l'ensemble des métabolites d'une cellule ou d'un organisme). Dans le cadre de l'étude de la réponse au stress induit par un toxique, ces approches permettent d'obtenir de nombreuses informations sur les réseaux de gènes spécifiquement activés ou inhibés, et les voies de signalisation impliquées. En particulier, les techniques de protéomique offrent une approche complémentaire à la transcriptomique pour

étudier les effets d'une exposition à une subtance toxique. En effet, l'analyse transcriptomique ne reflète pas toujours le profil d'expression de la protéine correspondante, et le niveau d'expression d'une protéine peut varier en fonction d'une modification de son environnement, due par exemple à un traitement. Le protéome représente, à un instant donné, l'ensemble des protéines ainsi que les formes ayant subi des modifications post-traductionnelles dans un système cellulaire. C'est pourquoi, dans notre projet, nous avons choisi ce type d'approche pour étudier plus finement les mécanismes de réponses des cellules hépatiques impliqués suite à une exposition aux mycotoxines.

Chapitre II : RESULTATS

Partie I : Evaluation de l'immunotoxicité aiguë des fusariotoxines seules et en mélange sur la lignée monocytaire humaine THP-1

Etude n°1

Effects of fusariotoxin co-exposure on THP-1 human immune cells

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Cette première partie avait pour objectif de mieux caractériser les effets toxicologiques induits par l'exposition et la co-exposition aux fusariotoxines sur les cellules immunitaires humaines THP-1. En particulier, les effets de six fusariotoxines seules (DON, MON, FB1, ZEA, NIV et T2) et en mélange (DON+MON, DON+FB1, DON+ZEA et NIV+T2), ont été évalués sur la viabilité et les mécanismes impliqués dans la mortalité cellulaire des THP-1 cultivées à l'état de monocytes. Comme expliqué précédemment, la lignée cellulaire THP-1 est aujourd'hui connue pour être un modèle simplifié, adapté et fiable dans l'étude des fonctions/réponses des monocytes et macrophages, de la différentiation des monocytes en macrophages et des effets possibles des stimuli externes du milieu environnant (Chanput et al., 2014).

Dans un premier temps, la toxicité de chaque fusariotoxine a été testée individuellement, après 48h d'exposition, pour une large gamme de concentrations *via* l'utilisation d'un test évaluant l'activité mitochondirale des cellules : le MTS (Mitochondrial Tetrazolium Salt assay) afin de déterminer les différentes concentrations inhibitrices de chaque mycotoxine (CI₁₀, CI₃₀, CI₅₀). Suite à l'obtention de ces valeurs, la toxicité des mélanges a pu être testée de la même façon (c'est-à-dire sur 48h *via* un test MTS) en utilisant des concentrations induisant la même toxicité pour chacune des mycotoxines du mélange. Plus précisément, dans l'exemple du mélange DON+ZEA, après avoir sélectionné 5 concentrations pour le DON, le ratio des CI₅₀ a été utilisé pour calculer les concentrations de la ZEA en mélange avec le DON. Ainsi, la CI₅₀ du DON étant 20 fois plus faible que celle induite par la ZEA, toutes les

concentrations utilisées pour la ZEA sont 20 fois plus élevées que celles du DON. La cytotoxicité induite par ces mélanges et par les toxines seules a ensuite pu être comparée en termes d'additivité, d'antagonisme ou de synergisme *via* le modèle mathématique appliqué par Weber et al. (2005) et beaucoup d'autres, et basé sur la définition arithmétique de l'additivité.

Dans un second temps, afin d'étudier les effets des fusariotoxines sur le phénotype des monocytes THP-1, l'expression des récepteurs de surface cellulaire CD14 et CD71 a été mesurée sur les cellules viables après une exposition de 48h aux fusariotoxines seules et en mélange, et à des concentrations cytotoxiques importantes (CI₅₀). Ces deux marqueurs de surface sont exprimés à des degrés divers sur les monocytes et les macrophages.

Les mécanismes précoces de mort cellulaire – tels que l'apoptose et la nécrose – ont ensuite été étudiés après 3, 6, 12 et 18h d'exposition, toujours aux CI₅₀ (obtenues après 48h d'exposition), *via* un double marquage des cellules à l'annexine V-FITC/iodure de propidium, ainsi qu'un marquage au DiOC6(3) suivie d'une analyse par cytométrie de flux. Le double marquage à l'annexine V-FITC/iodure de propidium permet de distinguer les cellules en apoptose précoce de celles en nécrose ou en apoptose plus tardive, alors que le DiOC6(3) permet d'identifier les cellules en apoptose imduisant une modification du potentiel mitochondiral et impliquant la voie intrinsèque.

Enfin, dans le but de mieux comprendre les effets de ces fusariotoxines sur les mécanismes cellulaires, l'expression et l'activation des principales MAPKs impliquées dans les cascades de signalisation contrôlant la prolifération, la différenciation, l'inflammation ou encore l'apoptose des cellules, à savoir p38, SAPK/JNK et ERK1/2, ont été mesurées. Plus particulièrement, les MAPKs p38 sont des régulateurs clés de la réponse inflammatoire des cellules, alors que les MAPKs SAPK/JNK sont davantage impliquées dans le contrôle de l'apoptose, et ERK1/2 dans celui de la croissance, la prolifération et la différentiation cellulaire (Johnson and Lapadat, 2002). Pour nos analyses, nous avons choisi un temps d'exposition court (1h) et des concentrations, cette fois, faiblement cytotoxiques (CI₁₀ obtenues après 48h), puisqu'il a pu être montré que ces MAPKs pouvaient être phosphorylées en moins d'une heure après la stimulation du stress cellulaire.

La figure 5 schématise de façon synthétique les principaux effets biologiques évalués dans cette étude.



Figure 5 : Résumé schématique des différents effets biologiques et conditions évalués dans l'étude n°1.

ORIGINAL ARTICLE



Effects of fusariotoxin co-exposure on THP-1 human immune cells

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Abstract Deoxynivalenol (DON), nivalenol (NIV), T-2 toxin (T2), fumonisin B1 (FB1), zearalenone (ZEA), and moniliformin (MON) mycotoxins are common food and feed contaminants produced by Fusarium spp. However, while they are usually found to co-occur in a large range of commodities, only few data are available on mycotoxin co-exposure effects and cellular response mechanisms. In this study, the individual and combined toxic effects of these fusariotoxins were evaluated on the THP-1 human immune cell line as major fusariotoxins are mostly potent immunomodulators. In particular, four relevant fusariotoxin mixtures, namely DON-MON, DON-FB1, DON-ZEA, and NIV-T2, were studied using several parameters including cell viability as well as the expression of cell surface markers and the main mitogen-activated protein kinases (MAPKs). After 48 h exposure, a reduction of cell viability in a dosedependent manner was observed for T2, the most cytotoxic mycotoxin, followed by NIV, DON, MON, FB1, and ZEA. Regarding mycotoxin mixtures, they mainly showed antagonism on cell viability reduction. Interestingly, at concentrations inhibiting 50% of cell viability, most viable cells exhibited surface marker loss and thus

M.-C. Smith · S. Madec · S. Troadec · E. Coton · N. Hymery (🖾) Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne, IBSAM, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France e-mail: nolwenn.hymery@univ-brest.fr became potentially non-functional. In addition, during the first 18 h of exposure, the effects of mycotoxin mixtures on early cell apoptosis and necrosis were found to be different from those induced by the toxins alone. At the molecular level, after 1 h exposure of individual and combined mycotoxins, the three main MAPK signaling pathways (p38, SAPK/JNK, and ERK1/2) were activated, highlighting a fast reaction of the exposed cells even at low cytotoxicity levels.

Keywords Co-exposure · Cytotoxicity · Fusariotoxins · MAP kinases · Monocytes · THP-1

Abbreviations

BEA	Beauvericin
DiOC6(3)	3,3'-Dihexyloxacarbocyanine iodide
DON	Deoxynivalenol
ENNs	Enniatins
FB1	Fumonisin B1
IC ₅₀	Inhibitory concentration 50%
MAPKs	Mitogen-activated protein kinases
MON	Moniliformin
MTP	Mitochondrial transmembrane potential
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-
	carboxymethoxyphenyl)-2-
	(4-sulfophenyl)-2H-tetrazolium
NIV	Nivalenol
PS	Phosphatidylserine
T2	T-2 toxin
TCT	Trichothecenes
ZEA	Zearalenone

Introduction

Cereals and cereal products are the most important human food and livestock feed resources in the world. These matrices are susceptible to preharvest (in the field) or postharvest (during storage) fungal contamination which has both a quality and a safety impact, as some species are mycotoxin producers. In the northern temperate regions of the world, Fusarium spp. are the most problematic species due to their prevalence, ecology, physiology, and ability to produce a wide range of mycotoxins (called fusariotoxins) (Smith et al. 2016; Yazar and Omurtag 2008). Three fusariotoxin families are particularly important because of their high toxicity and their occurrence in European agricultural products: trichothecenes (TCT) (mainly T-2 toxin, deoxynivalenol (DON), and nivalenol NIV), fumonisins (FUM), and zearalenone (ZEA). Moreover, several Fusarium species can produce the beauvericin (BEA), enniatins (ENNs), and moniliformin (MON) emerging mycotoxins (Jestoi 2008).

A 3-year survey on the worldwide occurrence of major mycotoxins in feed reported that 59, 45, and 64% of the 7049 analyzed samples were contaminated by DON, FUM, and ZEA, respectively (Rodrigues and Naehrer 2012). In 2016, another report carried out on 16,511 worldwide grain samples highlighted similar percentages of contaminations for these mycotoxins (BIOMIN 2016). In addition, the same survey showed that, among the 1378 cereal samples analyzed by a LC-MS/MS method targeting about 380 fungal metabolites, MON was one of the most distributed mycotoxins with higher concentrations on average than BEA or ENNs, contaminating 75% of the samples and more particularly 94% of the corn samples.

Concerning TCT, a family of about 150 metabolites, T-2 toxin (T2) appears to be one of the most cytotoxic ones in animal studies, having an immunosuppressive effect and causing a wide range of gastrointestinal, dermatological, and neurologic symptoms (Bennett and Klich 2003; Bouaziz et al. 2013). This mycotoxin has been retrospectively associated with human foodborne toxicosis known as alimentary toxic aleukia, characterized by a severe leukopenia with immune cell depletion and bone marrow aplasia (Yang et al. 2000). Regarding DON, another TCT family member, its consumption causes feed refusal and weight loss at low doses, whereas it induces nausea, vomiting, and diarrhea at higher acute doses. At the cellular level, DON was shown to significantly alter humoral immunity, cellmediated immunity, and host resistance in a variety of experimental animal models (Cetin and Bullerman 2005). Still within the TCT family, NIV was shown to disrupt the immune system by inhibiting blastogenesis in cultured human lymphocytes (Marzocco et al. 2009). Within the FUM group, fumonisin B1 (FB1) is the most abundantly produced and its consumption is associated with cases of esophageal cancer in human. Regarding ZEA, this non-steroidal estrogenic mycotoxin is usually non-lethal to animals but its estrogenicity has functional and morphological effects on reproductive organs (Zinedine et al. 2006). As for MON, its toxicological effects are currently poorly documented compared to other fusariotoxins. Nevertheless, in vivo studies showed the cardiotoxicity of MON and some in vitro studies highlighted the cytotoxicity of MON for human lymphocytes (Ficheux et al. 2012a).

From the regulatory point of view, regulated mycotoxins have been established on the basis of one individual evaluation, although the co-occurrence of mycotoxins is the common situation not the exception (Alassane-Kpembi et al. 2016; Smith et al. 2016). Furthermore, multi-exposures may lead to additive, synergistic, or antagonistic toxic effects, and the toxicity of the mixture cannot always be predicted based upon the individual toxicity of each mycotoxin from the mixture.

Since data concerning the effects of mycotoxin combined exposure on the immune system are still limited, the first aim of this study was to assess the toxicological combined effect of some major fusariotoxins present in food and feed (namely DON, NIV, T2, FB1, ZEA, and MON) towards human monocytes using the THP-1 model cell line. Molecular mechanisms induced by exposure to these fusariotoxins were also studied by targeting specific signaling pathways.

Materials and methods

Mycotoxins

DON standard (CAS no. 51481-10-8) with purity > 98%, MON standard (CAS no. 71376-34-6) with purity > 98% and produced from *Fusarium proliferatum*, FB1 standard (CAS no. 116355-83-0) with purity > 98% and produced from *Fusarium moniliforme*, ZEA standard (CAS no. 17924-92-4) with purity > 99%, as well as T2 standard (CAS no. 21259–

20-1) with purity > 98% and produced from *Fusarium* sp. were purchased from Sigma-Aldrich (St. Louis, MO, USA). NIV standard (CAS no. 23282-20-4) with purity > 99% was obtained from Oskar-Tropitzsh e.K. (Marktredwitz, Germany). The standards were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and stored at – 20 °C. The final concentration of solvent in the cell culture was 2% maximum. Appropriate control cultures without mycotoxin but containing the same amount of solvent were included as negative controls in each experiment.

Cell and culture conditions

Human monocytic leukemia cells (THP-1) were acquired from the European Collection of Cell Cultures (ECACC; no. 88081201, Salisbury, UK). THP-1 suspensions were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin 10,000 units/ mL, and 1% streptomycin 10,000 μ g/mL (Biochrom GmbH, Berlin, Germany) at 37 °C with 100% relative humidity (RH) in a 5% CO₂ atmosphere. Cells were grown to a density between 0.2 and 1 × 10⁶ cells/mL as recommended by ECACC. The culture medium was replaced every 3 days with fresh growth medium.

Mycotoxin exposure

Cytotoxicity assay by MTS test

To explore the individual and combined cytotoxic effects of DON, MON, FB1, ZEA, NIV, and T2 on THP-1 cells, mycotoxins dissolved in DMSO were added to the culture medium, followed by incubation at 37 °C for 48 h in 96-well plates. The highest tested concentrations for each mycotoxin corresponded to their solubility limits. Concerning the mixtures, the tested binary combination ratios were 1/10 for DON/MON, 1/12.5 for DON/FB1, 1/20 for DON/ZEA, and 1/0.0075 for NIV/ T2. These ratios, calculated from preliminary individual cytotoxicity experiments, enabled to obtain a similar toxicity for each toxin, based on their IC₁₀ values for DON-MON and DON-FB1, as well as inhibitory concentration 50% (IC₅₀) values for DON-ZEA and NIV-T2. These inhibitory concentrations (concentrations inhibiting 10 and 50% of cell viability from the negative control, respectively) were obtained from theoretical dose-response curves established with Microsoft TableCurve 2D Software v.5.01 (Microsoft, Redmond, WA, USA). Dose-response curves were obtained by plotting the percentage of cell viability against the log of mycotoxin concentrations.

Mycotoxin cytotoxicity towards THP-1 lineage cells was evaluated using Promega CellTiter 96AQueous Non-Radioactive cell proliferation assay (Promega, Madison, WI). This colorimetric method determines mitochondrial activity based on the reduction of a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium assay (MTS)) by mitochondrial dehydrogenases in viable cells. After 48 h incubation in the presence of the mycotoxins alone or in combination under normal incubation conditions, the culture media were removed and cells were washed with PBS. Then, 100 µL PBS was added in each well as well as 20 µL CellTiter 96AQueous Non-Radioactive solution and the cells were further incubated for 3 h. Formazan (MTS metabolite) was then quantified by reading the absorbance at 490 nm on a Multiskan FC plate reader (Thermo Scientific, Madison, WI). Each experiment was performed with biological and technical triplicates.

Immunofluorescence analysis of the CD14 and CD71 cell surface markers by flow cytometry

The THP-1 monocyte phenotype was investigated after 18 and 48 h of mycotoxin single and combined exposures using the immunofluorescence analysis of the CD14 and CD71 surface markers. To do so, THP-1 cells $(5 \times 10^5$ cells) were treated with DON, ZEA, NIV, and T2 at their respective IC₅₀ (1.8, 36.3, 0.8, and 0.006 μ M, respectively) or at solubility limits for MON and FB1 (10 μ M). Following the manufacturer's instructions (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), the cells were washed twice with PBS and resuspended in 100 µL PBS before a 20-min incubation period with FITC-conjugated mouse anti-human CD14 and PE mouse anti-human CD71 (both purchased from BD) in the dark at room temperature. After washing with 400 µL PBS, the cells were resuspended again with 400 µL PBS and analyzed by flow cytometry using a BD Accuri C6 flow cytometer associated with the BD Accuri C6 software (BD). FITC-fluorescence (FL-1) was collected through a 530-nm bandpass filter and PE mouse anti-human CD71 fluorescence (FL-2) through a 585-nm bandpass filter. Compensation for spectral overlap between FL-1 and FL-2 channels was performed using unlabeled and single stained cell populations. Three independent experiments were performed for each condition and 10,000 events were obtained for each sample. The isotype-matched mAb-stained cells were used as the background control in all experiments.

Cell mortality mechanism assessment by annexin V-FITC/propidium iodide cell double staining and DiOC6(3) assays followed by flow cytometry

An investigation of cell mortality mechanisms, such as apoptosis or necrosis, was performed on THP-1 cell cultures after mono- or co-exposure to DON, ZEA, NIV, and T2 at their respective IC_{50} or at solubility limits for MON and FB1.

The measurement of viable, apoptotic, and necrotic cell numbers after 3, 6, 12, and 18 h of mycotoxin exposures was performed by annexin V-FITC/ propidium iodide cell double staining (Miltenyi Biotec, Bergisch Gladbach, Germany), whereas the assessment of mitochondrial transmembrane potential (MTP) was measured using 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) (Sigma-Aldrich), after 6 h of mycotoxin exposures. Regarding annexin V-FITC/propidium iodide cells double staining and following the manufacturer's instructions, the cell cultures (5 \times 10⁵ cells/mL) were harvested at the end of the incubation period and then washed and suspended in 100 μ L 1X binding buffer followed by addition of 10 µL of annexin V-FITC. After homogenization, the cells were incubated in the dark at room temperature for 15 min and then washed in 1X binding buffer. Finally, cell pellets were resuspended in 500 μ L of 1X binding buffer and 5 μ L of propidium iodide was added prior to flow cytometry analysis. As described for the surface marker immunofluorescence analysis ("Immunofluorescence analysis of the CD14 and CD71 cell surface markers by flow cytometry" section), flow cytometry analysis was performed using a BD Accuri C6 flow cytometer. Propidium iodide fluorescence (FL-3) was collected through a > 670-nm bandpass filter. Compensation for spectral overlap between FL-1 and FL-3 channels was performed using unlabeled and single stained cell populations. Annexin V-positive and PI-negative cells were considered to be apoptotic cells in the early stage, whereas annexin V- and PI-positive cells were considered to be necrotic or apoptotic cells in the late stage.

Concerning the DiOC6(3) assay, this method allows to distinguish viable cells from apoptotic cells involving the intrinsic mitochondrial pathway. According to the manufacturer's instructions, 40 nM DiOC6 was added in the cell cultures 30 min before harvest. Then, THP-1 $(2 \times 10^5$ cells/mL) were harvested and washed twice with PBS. Cell pellets were finally suspended in 500 µL PBS prior to flow cytometry analysis where only FL-1 was collected. DiOC6(3)-positive cells were considered to be viable cells.

For both assays, three independent experiments were performed for each condition and used for cell mortality mechanism assessment.

p38 MAPK, ERK1/2, and SAPK/JNK activation assessment by western blotting

For the detection of total and phosphorylated mitogen-activated protein kinase (MAPK) p38, ERK1/2 and SAPK/JNK expression, 3×10^6 cells, were treated with DON, MON, FB1, ZEA, NIV, and T2 alone and in combination (DON-MON, DON-FB1, DON-ZEA and NIV-T2) at low cytotoxic doses, namely IC₁₀ (0.06 µM, 0.6 µM, 0.75 µM, 1.8 µM, 0.01 µM, and 1.1 nM, respectively) during 1 h. Afterwards, THP-1 cells were washed twice with cold PBS and lysed with 100 µL of ice-cold cell lysis buffer (NP40 Cell Lysis Buffer, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1 mM PMSF and protease inhibitor cocktail tablet (Complete EDTA-free, Roche Diagnostics, Mannheim, Germany). After 30 min on ice, with vortexing at 10 min intervals, cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. The clear lysates were transferred in clean microfuge tubes and protein concentrations were determined using the colorimetric assay Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). For each sample, 20 µg of total protein was denatured by boiling at 99 °C for 10 min with a 1:4 dilution of 4× Laemmli sample buffer (0.06 M Tris-HCl pH 6.8, 20% v/v SDS 10%, 10% v/v glycerol, 5% v/v bromophenol blue, and 5% v/vβ-mercaptoethanol in distilled water), completed with distilled water to 20 µL. Each sample was entirely dropped onto a 12% polyacrylamide gel for the separation of total proteins and run at 80 mA (Bio-Rad Mini-PROTEAN 3 Cell Electrophoresis System, Bio-Rad Laboratories, Hercules, CA, USA). Then, following the manufacturer's instructions (Cell

Signaling Technology, Danvers, MA, US), a transfer onto a nitrocellulose membrane (0.45 µm, GE Healthcare-Amersham, Buckinghamshire, UK) was conducted at 250 mA for 1 h. Membranes were saturated during 1 h with washing buffer trisbuffered saline (TBS)-0.1% Tween 20 (Sigma-Aldrich) containing 5% w/v non-fat dry milk. Membranes were then probed overnight at 4 °C with the appropriate primary antibody diluted in TBS-0.1% Tween 20 containing 5% BSA or non-fat dry milk (1:1000 for antibodies against total and phosphorylated MAPKs). All anti-active polyclonal antibodies (p38 MAPK, phospho-p38 MAPK, p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (phospho-Erk1/2), SAPK/JNK, phospho-SAPK/JNK, and β-actin) were purchased from Cell Signaling (Danvers, MA, USA) and stored at - 20 °C. Membranes were then incubated 1 h with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling) (diluted 1:2000 in TBS-0.1% Tween 20 and 5% non-fat dry milk). Band detection was performed with the ECL Prime Western Blotting Detection Reagent (GE Healthcare-Amersham, Buckinghamshire, UK) and membranes were scanned using the G-Box

system (Syngene, Cambridge, UK). Three independent experiments were performed and blots were analyzed using the Gene Tools analysis software (Syngene, Cambridge, UK). The relative protein expression levels were normalized to β -actin expression and to total protein form. The results were represented as fold change relative to the level expressed in untreated control cells.

Statistical analysis

For MTS assays, cell viability mean percentages of three independent experiments \pm standard error of mean (SEM) were used for statistical analyses. The cell viability obtained for the negative control was defined as 100%. The results obtained from MTS co-exposure experiments were analyzed following the model used by Weber et al. (2005). This method is based on the comparison of theoretical expected values calculated on the basis of mono-exposure experiment results with actual values obtained from MTS co-exposure experiments. In the case of binary exposures, the expected values were calculated as follows:

Cell viability expected value for *mycotoxin* 1 + mycotoxin 2 (%) = mean cell viability for*mycotoxin*<math>1 (%) + mean cell viability for*mycotoxin*<math>2 (%) - mean control condition (100%)

and the expected SEM was calculated as follows:

SEM expected for mycotoxin 1 + mycotoxin 2

$$= \left[(SEM \text{ for mycotoxin } 1)^2 + (SEM \text{ for mycotoxin } 2)^2 \right]^{1/2}$$

The combined cytotoxic effects were determined by comparing each cell viability expected value and the corresponding measured mean value obtained from co-exposition experiments, using an unpaired *t* test and appropriate tables according to the following equation (*P* values < 0.05) (Weber et al. 2005):

t test value =
$$\left| mean_{measured} - mean_{expected} \right|$$

 $\left/ \left[(SEM_{measured})^2 + (SEM_{expected})^2 \right]^{1/2} \right|$

No statistical difference between expected and measured mean values was interpreted as an additive

effect on cell viability reduction. If the measured mean value was significantly lower than the expected values, the results were interpreted as a synergistic effect. On the contrary, when the measured mean value was significantly higher than the expected values, it was associated with an antagonistic effect.

For apoptosis and necrosis assays (annexin V-FITC/ propidium iodide double staining and DiOC6 staining), the means of viable, apoptotic, and necrotic cell populations of three independent experiments \pm SD were used for statistical analyses, which were performed using Statistica for Windows (version 10; StatSoft). After verifying normal data distribution and variance homogeneities, different mean value groups were compared to control values and to other exposure period according to the least significant difference (LSD) test of multifactor ANOVA analysis followed by HSD Turkey's post hoc test (*P* values < 0.05).

Results

Cell viability assessment

After 48 h, mycotoxin single exposures of monocytic cell cultures led to a cell viability reduction in a dosedependent manner (Fig. 1). More precisely, these fusariotoxins exerted clear toxicity at concentrations above 0.025 μ M for T2, 0.01 μ M for NIV, 1 μ M for DON and FB1, and 10 µM for MON and ZEA. THP-1 viability decreased by more than 90% compared to control at 1 µM for T2, 10 µM for NIV and DON, and 100 μ M for ZEA (subpanels a, b, c, and d of Fig. 1, respectively). The IC₅₀ of T2, NIV, DON, and ZEA were 0.0058, 0.77, 1.82, and 36.31 µM, respectively (Table 1). Regarding MON and FB1, after 48 h exposure at the highest dose, namely 10 µM (solubility limit in DMSO), cell viability only decreased by about 30 and 40%, respectively (Fig. 1e, f); thus, MON and FB1 IC_{50} could not be determined.

For each mycotoxin mixture, five combinations were tested (Fig. 2). As stated above, the selected DON concentrations (namely 0.1, 0.8, 2, 4, and 10 μ M) were

Fig. 1 Effect of a T2, b NIV, c DON, d ZEA, e MON, and f FB1 on THP-1 cells after 48 h exposure (mean percentage \pm SD of cell viability quantified using MTS bioassay, N = 3). *Plus sign*: mean measured cell viability significantly different from control (100%) (P < 0.05)

Table 1	I IC ₁₀ , IC	30, and IC	o for T2	2, NIV, DO	N, ZEA, M	ION, and
FB1 re	sulting in	10, 30, ai	nd 50%	inhibition	of THP-1	viability
after 48	3 h exposi	re (calcula	ted usir	ng TableCu	rve 2D sof	tware)

Mycotoxin	IC ₁₀	IC ₃₀	IC ₅₀
T2	1.10 nM	2.26 nM	5.78 nM
NIV	0.01 µM	0.32 μM	0.77 μM
DON	0.06 µM	0.52 μM	1.82 µM
ZEA	1.76 μM	16.01 μM	36.31 µM
MON	0.61 µM	$> 10 \ \mu M^a$	$> 10 \ \mu M^{a}$
FB1	0.75 μΜ	5.27 µM	> 10 µM ^a

^a Highest tested concentration

multiplied by 10, 12.5, and 20 to obtain MON, FB1, and ZEA doses, respectively. However, for MON and FB1, 10 μ M (solubility limit in DMSO) was used in combination with DON concentrations above 1 μ M. Concerning NIV-T2 mixture, NIV concentrations (namely 0.01, 0.3, 0.8, 3, and 10 μ M) were multiplied by 0.0075 to obtain T2 doses.

The combined exposure of THP-1 cells at the lowest DON-MON (0.1 μ M DON/1 μ M MON and 0.8 μ M DON/8 μ M MON) and DON-FB1 (0.1 μ M DON/





Fig. 2 Effect of a DON-MON, b DON-FB1, c DON-ZEA, and d NIV-T2 co-exposure on THP-1 cell viability after 48 h exposure (dark gray = measured mean percentage \pm SEM of cell viability using MTS bioassay, N = 3; light gray = expected cell viability \pm SEM calculated using the model described by Weber et al.

1.25 µM FB1) concentrations did not show significant cytotoxic effects compared to the negative control (Fig. 2a, b). Significant cytotoxicity was observed at higher concentrations for these mixtures, whereas DON-ZEA and NIV-T2 mixtures showed significant cytotoxic effects for all tested combinations (Fig. 2c, d). Comparing the calculated expected values and the measured ones obtained from 48 h co-exposure experiments according to Weber et al. (2005), additive and antagonistic effects on cell viability reduction were observed (Fig. 2). More particularly, DON-MON co-exposure led to additivity at the lowest concentration and antagonism at higher doses (Fig. 2a), whereas DON-FB1 and DON-ZEA co-exposure induced antagonistic cytotoxic effects for all tested combinations (Fig. 2b, c). For NIV-T2 co-exposure, additivity on cell viability reduction was observed at the two lowest doses and antagonism at higher doses (Fig. 2d).

Effects on monocyte surface marker expression

To characterize possible effects of mycotoxins on monocyte function, the expression of the CD14 and CD71 cell surface markers was evaluated by flow cytometry after



(2005)). *Plus sign*: measured mean cell viability significantly different from control (100%) (P < 0.05). *Asterisk*: measured mean cell viability significantly different from expected cytotoxicity (antagonistic effect) (P < 0.05)

18 and 48 h of mycotoxin exposure. The results showed that all mycotoxins and combinations seemed to significantly induce down-regulation of CD14 and CD71 after both 18 and 48 h exposure (Fig. 3). More particularly, after DON and T2 single exposures, THP-1 cells seemed to totally lose their CD71 surface marker after 18 h exposure, whereas only T2 seemed to inhibit CD14 expression after 48 h exposure. Regarding the mixtures, they seemed to totally inhibit CD71 expression after 18 and 48 h exposure, whereas CD14 expression after 18 negosure, whereas CD14 expression was interrupted after 48 h exposure only.

Mortality mechanisms associated with mycotoxin exposures

Annexin V-FITC/propidium iodide cell double staining followed by flow cytometry analysis allowed to explore early cell mortality mechanisms, namely early apoptosis and necrosis after mycotoxin single and combined exposures. Depending on the condition, after DON, MON, FB1, ZEA, NIV, and T2 mono and co-exposures of THP-1 cells (at IC_{50} or solubility limits), quantitative variations of apoptotic and necrotic cells were observed at 3, 6, 12, and 18 h (Fig. 4a, b, c, d).



Fig. 3 Effect of 1.8 μ M DON, 10 μ M MON, 10 μ M FB1, 36.3 μ M ZEA, 0.8 μ M NIV, and 0.006 μ M T2 alone and in combination on **a** CD71 expression and **b** CD14 expression after

The results after 3 h of mycotoxin single and combined exposures showed that only DON-FB1 and DON-ZEA mixtures significantly reduced cell viability compared to the control condition and that by inducing mainly early cell apoptosis (P < 0.05) (Fig. 4a). As for 6 h exposure conditions, only T2 and the DON-FB1 combination appeared to significantly reduce cell viability, mostly by inducing early cell apoptosis



Fig. 4 Effect of 1.8 μ M DON, 10 μ M MON, 10 μ M FB1, 36.3 μ M ZEA, 0.8 μ M NIV, and 0.006 μ M T2 alone and in combination on THP-1 cell viability as well as early apoptosis and necrosis after a3 h, b 6 h, c 12 h, and d 18 h exposure (mean percentage ± SD of necrotic cells, early apoptotic cells, and viable



18 and 48 h exposure (mean percentage of control \pm SD, N = 3). *Plus sign*: mean measured value significantly different from control (100%) (P < 0.05)

(P < 0.05), in comparison to the control (Fig. 4b). After 12 h of exposure, cell viability was significantly reduced by T2 single exposure as well as by DON-FB1, DON-ZEA, and NIV-T2 mixture exposures (P < 0.05) (Fig. 4c). Concerning T2 and the NIV-T2 mixture, mainly cell necrosis was induced after 12 h of exposure, whereas DON-FB1 mixture induced mainly early cell apoptosis. The two cell death mechanisms seemed to be involved



□Viable cells □Early apoptotic cells □Late apoptotic or necrotic cells

cells quantified using annexin V-FITC/propidium iodide double staining bioassay, N = 3). *Plus sign*: mean measured cell viability significantly different from control (P > 0.05 ANOVA analysis followed by HSD Turkey's post hoc test)

in the cell viability reduction after 12 h of DON-ZEA combined exposure. Finally, after 18 h of exposure, the results showed that FB1, ZEA, and T2 single exposures as well as DON-FB1, DON-ZEA, and NIV-T2 combined exposures reduced significantly THP-1 viability (P < 0.05) (Fig. 4d). Regarding cell death mechanisms associated with cell viability reduction, mainly cell necrosis was induced by T2 and NIV-T2, whereas early cell apoptosis was primarily induced by FB1 and both mechanisms were simultaneously induced by ZEA, DON-ZEA, and DON-FB1.

Thus, as for DON and MON alone, the DON-MON combination did not significantly induce early apoptosis or/and necrosis at the tested exposure durations. On the contrary, FB1, ZEA, NIV, and T2 as well as DON-FB1, DON-ZEA, and NIV-T2 mixtures were found to significantly reduce cell viability compared to control. However, for these mixtures, different cell death mechanisms were involved in comparison to the single toxin exposures. Moreover, concerning DON-FB1 and DON-ZEA combinations, cell viability reduction was observed from 3 h of exposure whereas cytotoxicity was only noticed from 18 h of exposure for FB1 and ZEA single exposures.

Mitochondrial transmembrane potential measurement

DiOC6(3) cell staining assay provides information on early cell apoptosis by measuring cell MTP. After 6 h single exposure of THP-1 cells to DON, MON, FB1, ZEA, NIV, and T2, at their respective IC_{50} or solubility limits, only DON significantly reduced cell viability compared to the control (P < 0.05). Indeed, in this case, the number of viable cells was reduced by about 1.7fold compared to the control condition (Fig. 5). Regarding the tested mixtures, DON-MON, DON-FB1, and DON-ZEA significantly reduced cell viability compared to the control (between 1.7 and 2-fold depending on the combination). Thus, the tested mixtures seemed to act in the same way on the MTP as the toxins alone. In fact, the mixtures involving DON modified THP-1 MTP similarly to DON alone and NIV-T2 combination did not significantly affect the MTP as for NIV and T2 alone.

Effects of mycotoxin exposure on MAPK activation

For MAPK expression, THP-1 cells were studied after 1 h of single and combined exposures to DON, MON,



Fig. 5 Effect of 1.8 μ M DON, 10 μ M MON, 10 μ M FB1, 36.3 μ M ZEA, 0.8 μ M NIV, and 0.006 μ M T2 alone and in combination on THP-1 cell viability after 6 h incubation (mean \pm SD of viable cells quantified using DiOC6(3) cell staining, N = 3). *Plus sign*: mean measured cell viability significantly different from control (P > 0.05 ANOVA analysis followed by HSD Turkey's post hoc test)

FB1, ZEA, NIV, and T2 at low cytotoxicity levels (IC_{10}) (0.06 µM, 0.6 µM, 0.75 µM, 1.8 µM, 0.01 µM, and 1.1 nM, respectively). The results clearly showed a phosphorylation of the three main MAPK signaling pathways, namely p38, SAPK/JNK, and ERK1/2, in both single and co-exposure conditions (Fig. 6). However, the level of phosphorylation activation was different depending on the considered mycotoxin or mixture. For all tested mycotoxins and combinations, a strong intensity of the band corresponding to the p38 MAPKphosphorylated form was observed in comparison to the control. Nevertheless, concerning mono-exposure conditions, DON seemed to induce more phosphorylation of p38, followed by MON, FB1, ZEA, and NIV and then T2. As for the tested mixtures, DON-ZEA seemed to activate the phosphorylated p38 MAPK more than other couples. Interestingly, the activation of the phosphorylated p38 form was less apparent with the DON-MON, DON-FB1, and DON-ZEA mixtures than with DON single exposure. In a similar way, NIV-T2 revealed a less intensive band of the phosphorylated p38 form than for NIV alone. Concerning SAPK/JNK, all the single fusariotoxins, especially ZEA and NIV, activated this signaling pathway. For the mixtures, the SAPK/JNK phosphorylation was also activated but showed more distinct results in comparison to the single exposures. Indeed, a decreased SAPK/JNK phosphorylation was observed in cells incubated with DON-ZEA and NIV-T2 in comparison to ZEA and NIV exposure alone. On the other hand, we observed a significant increase of phosphorylated SAPK/JNK in cells incubated with DON-MON and DON-FB1 in comparison to DON,

а	``									1012	B1 1	EA N	
MW (kD)	Controt	0014	MON	FBI	TEA	414	\sim	Contro	DOLY	D014	DOLY	MNIT	1
38	1	1	1	1		-	١	-	-	-	-	-	p38
38		-	-	-	_	_	-			1	-	-	Phosphorylated-p38
54 46	-	-	-			-	-	-				-	SAPK/JNK
46	1	-	_		_	-	_	-	_				Phosphorylated-SAPK/JNK
44 42	=	=	=	-	=	=	=	-	=	=	-	-	ERK 1/2
44 42		=	-	=	-	-	=	-	=	=	-	=	Phosphorylated-ERK 1/2
45	_	-	_	_	_	-	-	-	-	-	-	-	β-actin

Fig. 6 Expression of MAPKs induced by DON, MON, FB1, ZEA, NIV, and T2 alone and in combination: lysates (20 μ g) from THP-1 cells (3 × 10⁶ cells/mL) incubated in the absence (control) or presence of mycotoxin (IC₁₀) (0.06 μ M, 0.6 μ M, 0.75 μ M, 1.8 μ M, 0.01 μ M, and 1.1 nM, respectively) for 1 h were subjected to western blot analysis using total and phosphospecific p38

MAPK, SAPK/JNK, and ERK1/2 antibodies. **a** Presented immunoblots are representative of three experimental replicates. **b** Mean percentage \pm SEM of protein expression, N = 3. *Plus sign*: measured mean protein expression significantly different from negative control (arbitrarily set to 1) (P < 0.05)

MON, or FB1 alone. Regarding ERK1/2, the results were similar to those obtained with SAPK/JNK, except for MON which did not seem to activate the

phosphorylation of these MAPKs. All other individual fusariotoxins, especially ZEA and NIV, activated this signaling pathway. Moreover, all combinations showed



Fig. 6 continued.

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a similar activation of the phosphorylated ERK1/2 form. Nevertheless, compared to single exposure conditions, DON-MON and DON-FB1 mixtures induced more ERK1/2 phosphorylation than DON, MON, or FB1 alone, while DON-ZEA showed a similar activation than ZEA alone and NIV-T2 a less important activation than NIV alone.

Discussion

Nowadays, the involvement of fusariotoxins in many human and animal health disorders is well-established. However, while these mycotoxins are generally present simultaneously in food and feed, their combined effects are still little studied. A worldwide survey led by BIOMIN in 2016 reported the levels found on average in grain samples for about 380 fungal metabolites (BIOMIN 2016). In the latter report, DON, MON, FB1, ZEA, and NIV were found in 56, 75, 46, 63, and 42% of the analyzed samples, respectively. The reported average concentrations were 500, 86, 934, 64, and 114 µg/kg, respectively. Regarding T2, since it was found in less than 40% of the samples, its concentration was not provided. Nevertheless, part of this survey focusing on Europe showed that this mycotoxin was found in about 23% of the analyzed samples with an average concentration of 29 μ g/kg.

Most of these fusariotoxins are potent immunomodulators and have been found to disrupt human monocyte differentiation into macrophage (Ficheux et al. 2013; Hymery et al. 2009; Solhaug et al. 2016), a central part of the immune response. In order to study the immune effects of mycotoxins, different approaches can be used. The first one corresponds to the use of freshly isolated peripheral blood mononuclear cells. This approach allows to study normal cells; however, in order to take into account individual variability, the selection of several donors with a known medical history is necessary. Moreover, it does not allow for easy comparison between several studies worldwide. An alternative solution would consist in using a cancerous cell line. While these cells are not normal cells, they are easy to acquire and facilitate in vitro method standardization as well as inter-study comparison (Smith et al. 2016). In the context of this study, we chose to use the THP-1 human leukemia monocytic cell line. This cell line expresses characteristic markers of monocytes in suspension and is known to be a suitable and reliable model to study monocytes (Chanput et al. 2014). Furthermore, its sensitivity was shown to be similar to that of CD14⁺ cells (Fontaine et al. 2015) suggesting that it can be a relevant in vitro cell model to study mycotoxin immunotoxicity.

In this study, THP-1 cells appeared to be more sensitive to T2 > NIV > DON > MON > FB1 > ZEA, when tested individually. These results were consistent with literature data indicating, especially, that T2 was found to be a strong toxicant compared to other fusariotoxins, while DON was described as one of the weakest toxic TCT (Yazar and Omurtag 2008). Comparison with the doses used in the present study showed that, except for T2, all determined IC_{50} values were above the average concentrations found in cereals. However, regarding IC_{10} values, aside from ZEA, they all appeared to be below the average concentrations reported in the BIOMIN report. Furthermore, the maximum doses found in cereal samples for each toxin are higher than the concentrations used in the present study, as conversion of the BIOMIN reported average concentrations in micromolar were of 1.69, 0.72, 1.29, 0.2, 0.37, and 0.06 µM for DON, MON, FB1, ZEA, NIV, and T2, respectively. Thus, our results suggested that the doses found in grains worldwide present a low cytotoxicity.

Beyond the intrinsic toxicity of a given mycotoxin, its bioavailability could also influence the observed results. As stated by Groothuis et al. (2015), compounds may differentially and non-specifically bind to various elements (serum proteins, plastic of well plates...). In this study, we considered that in vitro kinetics probably did not significantly influence the analysis as suggested by the low log P (or log K_{OW}) values of the mycotoxins (i.e., -0.71, 1.84, 3.58, -2.24, and 2.27 for DON, FB1, ZEA, NIV, and T2, respectively), indicating that these compounds were not highly lipophilic and dissolved in the medium rather than bind to protein, lipid, or plastic.

Regarding the cytotoxic combined effects of mycotoxins, several experimental designs can be used. However, the simplest and most intuitive mathematical design to study mycotoxin interactions seems to be the arithmetic definition of additivity applied by Weber et al. (2005). In addition, since it is the most used method in studies investigating mycotoxin combined effects (about 30% of the papers), this model appears to be the most convenient for a proper inter-study comparison (Smith et al. 2016). Here, at high doses, all the tested binary combinations reduced cytotoxicity after 48 h of exposure compared to individual mycotoxin exposures. Interestingly, a cell count after 18 and 48 h of mycotoxin single and combined exposures showed a cell number reduction after 18 h exposure compared to 0 h, while an increase in cell number was observed after 48 h exposure, highlighting a proliferation of the viable cells between 18 and 48 h (data not shown). This observation could provide elements to elucidate the observed antagonism on cell viability reduction after mycotoxin combined exposure.

A comparison of our results with literature data showed that no in vitro study was conducted on the combined effect of DON-MON mixture, even if they are commonly encountered together in food products. Contrariwise, the combined cytotoxic effects of DON-FB1 mixture have already been studied by several authors (Ficheux et al. 2012b; Kouadio et al. 2007; Wan et al. 2013) and their conclusions varied considerably from a study to another. Nevertheless, our results appeared to be similar to those obtained by Ficheux et al. (2012b) in which human hematopoietic progenitors CFU-GM were used. In this study, antagonistic cytotoxicity at low doses of DON and FB1 (0.01-2 µM for DON and 0.5-2 µM for FB1) were observed after 14 days of exposure. However, by comparing the IC_{50} values obtained from the CFU-GM with those from THP-1 cells, the hematopoietic progenitors appeared to be more sensitive to fusariotoxins which could be explained by a difference in terms of differentiation state. Some authors have also studied DON-ZEA combination in in vitro conditions (Bensassi et al. 2014; Ficheux et al. 2012b; Kouadio et al. 2007; Wan et al. 2013) and the observed toxicological combined effects were very different depending on the study. Regarding the NIV-T2 mixture, only Thuvander et al. (1999) studied their in vitro combined effect on human lymphocyte proliferation after 72 h exposure and reported additivity on cell viability reduction at low concentrations as observed in the current study.

Today, the action mechanisms of the main fusariotoxins are partly elucidated. TCTs, including DON, NIV, and T2, are known to be potent eukaryotic protein synthesis inhibitors by binding to ribosomes (da Rocha et al. 2014). FUM, including FB1, are known to disrupt the sphingolipid metabolism (Soriano et al. 2005) while ZEA has a strong affinity to the cytosolic estrogen receptors and thus generally induces estrogenic toxicological effects (Kuiper et al. 1998; Kuiper-Goodman et al. 1987). Finally, MON is known to inhibit the pyruvate dehydrogenase (Gathercole et al. 1986) and consequently interferes with the Krebs cycle and oxidative phosphorylation (Thiel 1978). Thus, there is no obvious possible interaction between these mycotoxins, except maybe between TCT which present similar mechanisms and which could explain the antagonistic effect observed for NIV-T2 mixture.

To investigate monocyte phenotype, expression of the CD14 and CD71 cell surface receptors, allowing characterizing the differentiation process (Solhaug et al. 2016), was measured. Here, a down-regulation of the two cell surface receptors was observed for all mycotoxins tested individually while every mycotoxin mixture seemed to totally inhibit this receptor expression after 48 h of exposure. These findings raised the question of cell functionality even if 50% of the cell population was still alive. Some authors have also observed the modulation of the expression of cell surface markers by fusariotoxins in vitro in monocyte-derived dendritic cells obtained from pigs (Bimczok et al. 2007) or from humans (Hymery et al. 2006) as well as in macrophages from human blood monocytes (Waché et al. 2009).

Then, in order to elucidate cytotoxicity mechanisms, apoptosis and necrosis were studied at the IC₅₀, since it is known that one cell death mechanism can be preferentially involved depending on the considered toxin, which was confirmed by this study. In addition, the tested mixtures seemed to act differently from the toxins alone. Similarly, Ren et al. (2015) revealed that after 48 h exposure on chicken splenic lymphocytes, DON (0.674 to 168.5 µM) significantly induced apoptosis and necrosis in a dose-dependent manner compared to the control condition, apoptosis being the main induced cell death mechanism in this case. Nevertheless, a comparison with other studies is arduous as phosphatidylserine (PS) externalization during apoptosis is mainly cell line-dependent (Lee et al. 2013). Moreover, Geske et al. (2001) showed that the early stages of p53-induced apoptosis, such as PS externalization, might be reversible. Indeed, early during apoptosis, there is an externalization of PS in cell membrane as well as reduction in MTP which suggests the loss of mitochondrial membrane integrity. Ozgen et al. (2000) compared DiOC6(3) uptake and annexin V-propidium iodide co-labeling techniques in the quantification of early cell apoptosis using different cell lines and reported that MTP reduction and PS externalization may be common to many apoptotic pathways. Nevertheless, for

some cell lines, the percentage of apoptotic cells detected by the DiOC6(3) technique was higher than the rates determined by annexin V-propidium iodide, which may indicate repair and recovery, at least in some cells. In addition, even if externalization of PS in the cell membrane detected by annexin V binding occurs prior to nuclear condensation and DNA fragmentation, this event is probably posterior to the reduction of MTP in the apoptotic process (Castedo et al. 1996). Thus, these studies could explain the decrease of MTP after 6 h of exposure before the observed PS externalization at 12 or 18 h for DON-FB1 and DON-ZEA mixtures. Regarding DON and DON-MON combination, only MTP changes were induced; PS externalization could appear after 18 h of exposure. Indeed, data from the DiOC6(3) assay showed that only DON, as well as all the mixtures involving DON, seemed to induced early apoptosis. These results were consistent with those of Bensassi et al. (2014) who showed that DON incited permeabilization of mitochondrial membranes. The results obtained from MON, FB1, ZEA, NIV, T2, and NIV-T2 exposures showed that, even if apoptosis and/or necrosis were involved in cell mortality, the intrinsic apoptosis pathway was not necessarily involved. Other apoptotic pathways and/or other time of exposure could be selected to gain a better knowledge of the involved mechanisms.

Finally, in order to investigate the underlying mechanisms of early cell response to mycotoxin exposure, the expression and activation of MAPKs (p38, SAPPK/ JNK, and ERK1/2) after 1 h of mycotoxin single and co-exposures at the IC₁₀ were assessed. The phosphorylated forms of these MAPKs are effectors of signaling pathways. MAPKs are known to be phosphorylated in less than 1 h after cell stress stimulation. For instance, Pan et al. (2013) observed that DON at 0.8 μ M activated SAPK/JNK on RAW 264.7 murine macrophages from 5 min of exposure and after 15 min for p38 and ERK1/2, with a maximum effect from 30 min. In our study, an increase of the p38, SAPK/JNK, and ERK1/2 phosphorylated forms was observed and their level could vary depending on the considered mycotoxin or mixture. In addition, some mixtures seemed to reduce or enhance MAPK activation. For instance, p38 activation was significantly reduced when DON was associated to other fusariotoxins, such as MON or FB1, while SAPK/JNK and ERK1/2 activation was significantly reduced when DON was associated with ZEA, compared to ZEA alone. Thus, the activation of MAPKs by these fusariotoxins preceded the observed apoptosis. Our results were consistent with previous studies which showed the MAPK induction by mycotoxins, even if in the present study, very low cytotoxicity concentrations were used compared to the other authors (Baltriukiene et al. 2007; Pan et al. 2013; Pinton et al. 2012; Yang et al. 2000; Yu et al. 2011). Nevertheless, in order to determine if the MAPK activation is really involved in cell apoptosis induction for each toxin, it would be of interest to assess cell viability following an incubation period with MAPK-specific inhibitors. Here, our results suggested that these fusariotoxins have different molecular targets and then followed different signaling pathways to inhibit cell proliferation.

Conclusion

The present study demonstrated the high sensitivity of THP-1 cells to fusariotoxin exposure, even at very low concentrations. Moreover, antagonism was observed on cell viability after 48 h of fusariotoxin co-exposures, suggesting that the multi-exposure of fusariotoxins may be less or equally cytotoxic than the presence of mycotoxins alone. Different cell death mechanisms underlying these effects were observed, depending on the considered mycotoxin or mixture, highlighting interactions between fusariotoxins during co-exposure. The observed antagonistic behavior for all mixtures could be due to the mycotoxins competing for the same receptors. The greater toxicity and higher receptor affinity of DON compared to MON, FB1, and ZEA, and T2 compared to NIV, may result in the accumulation of the less toxic compound and a lower overall toxicity than would be predicted by the additive effect. This hypothesis is supported by the MAPK activation results highlighting that all tested fusariotoxins activated the same MAPKs and thus potentially disrupted the same biological pathways in the cell. The study of mycotoxin mixture cytotoxicity and the underlying molecular cell mechanisms should allow to better take into account the reality of food and feed mycotoxin contamination in quantitative risk assessment.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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Données supplémentaires : Caractérisation des effets cytotoxiques combinés via les modèles de Bliss (Bliss, 1939) et Chou-Talalay (Chou, 2006; Chou and Talalay, 1984)

Le modèle basé sur la définition arithmétique de l'additivité a longtemps été l'un des plus utilisés pour caractériser l'effet combiné des mycotoxines en termes d'antagonisme, d'additivité ou de synergisme (Smith et al., 2016). Cependant, ce modèle étant aujourd'hui débattu par une grande partie de la communauté scientifique, l'utilisation d'autres approches mathématiques telles que le modèle de Bliss (Bliss, 1939) (également connu sous le nom de « *response addition* » ou « *independent joint action* »), celui de Loewe (Loewe and Muischnek, 1926) (couramment appelé « *concentration addition* » ou « *dose addition* ») ou encore celui de Chou et Talalay (Chou, 2006; Chou and Talalay, 1984) (communément nommé « *median effect principle of the mass action law* » ou « *combination index-isobolographic analyis* »), se fait de plus en plus fréquente. En effet, la stratégie appliquée par Weber et al. (2005) suppose que les mycotoxines se comportent de façon à avoir un effet dose linéaire, ce qui n'est pas forcément le cas. L'exemple de l'étude de l'effet combiné de plusieurs doses de la même mycotoxine, qui ne peut pas être synergique ou antagoniste, met en évidence ce point.

Pour chacun de ces modèles, il est possible de calculer un indice de combinaison (IC), indiquant un effet supérieur (IC<0,9), inférieur (IC> 1,1) ou similaire (0,9 \leq IC \leq 1,1) à l'effet attendu. Cet indice est reconnu comme une mesure standard de l'effet combiné. Puisqu'il n'existe pas encore aujourd'hui de modèle de référence dans l'évaluation des effets combinés de différents composés, nous avons choisi d'en sélectionner plusieurs et de les comparer. En particulier, nous avons comparé les valeurs des IC obtenues avec la définition arithmétique de l'additivité (modèle utilisé dans notre étude, noté IC_{ADDITIVITE}), à celles calculées *via* les méthodes de Bliss (appelé IC_{BLISS}) et de Chou-Talalay (nommé IC_{CHOU-TALALAY}). Les calculs détaillés des IC_{ADDITIVITE} et IC_{BLISS} sont disponibles dans la récente revue de Foucquier and Guedj (2015) (sous les noms de « *response additivity* » et « *Bliss independence model* » respectivement). Pour le modèle de Chou-Talalay, les IC ont été générés avec le logiciel CompuSyn version 3.0.1 (ComboSyn, Inc., Paramus, NJ, 07652 USA, Chou et Martin 2005). Les valeurs des différents IC ont été reportées dans le tableau 5.

Tableau 5 : Valeurs des IC calculés selon le modèle de la définition arithmétique de l'additivité ($IC_{ADDITIVITE}$), selon celui proposé par Bliss (1939) (IC_{BLISS}) et selon celui développé par Chou et Talalay (1984) ($IC_{CHOU-TALALAY}$) en fonction de la combinaison de concentrations testée pour les mélanges DON+MON, DON+FB1, DON+ZEA et NIV+T2 sur les cellules HepaRG après 48h d'exposition.

Mélange		ICADDITIVITE	ICBLISS	ICCHOU-TALALAY
	$0,1 \ \mu M \ DON + 1 \ \mu M \ MON$	4,80 Ant	4,51 Ant	
	0,8 μM DON + 8 μM MON	2,00 Ant	1,70 Ant	
DON+MON	$2 \mu M DON + 10 \mu M MON$	1,55 Ant	1,26 Ant	NA*
	$4 \ \mu M \ DON + 10 \ \mu M \ MON$	1,51 Ant	1,21 Ant	
	$10 \mu\text{M}$ DON + $10 \mu\text{M}$ MON	1,40 Ant	1,11 Ant	
	0,1 μM DON + 1,25 μM FB1	2,89 Ant	2,70 Ant	
	0,8 μM DON + 10 μM FB1	2,11 Ant	1,72 Ant	
DON+FB1	$2 \mu M$ DON + $10 \mu M$ FB1	1,36 Ant	1,06 Add	NA*
	4 μM DON + 10 μM FB1	1,44 Ant	1,10 Add	
	10 μM DON + 10 μM FB1	1,48 Ant	1,09 Add	
	$0.1 \mu\text{M}$ DON + $2 \mu\text{M}$ ZEA	2,82 Ant	2,61 Ant	5,44 Ant
	0,8 μM DON + 16 μM ZEA	1,46 Ant	1,22 Ant	3,00 Ant
DON+ZEA	$2 \mu\text{M}$ DON + $40 \mu\text{M}$ ZEA	1,53 Ant	1,09 Add	1,32 Ant
	$4 \mu\text{M}$ DON + $80 \mu\text{M}$ ZEA	1,95 Ant	1,20 Ant	1,63 Ant
	10 µM DON + 100 µM ZEA	1,89 Ant	1,03 Add	0,23 Syn
	0,01 µM NIV + 0,075 nM T2	1,42 Ant	1,30 Ant	0,15 Syn
NIV+T2	0,3 μM NIV + 2,3 nM T2	1,33 Ant	1,16 Ant	1,74 Ant
	0,8 µM NIV + 6 nM T2	1,72 Ant	1,29 Ant	1,69 Ant
	3 µM NIV + 25 nM T2	1,96 Ant	1,21 Ant	1,94 Ant
	10 µM NIV + 75 nM T2	2,01 Ant	1,13 Ant	2,73 Ant

 $CI < 1, 0.9 \le CI \le 1.1$ and CI > 1.1 indique du synergisme (Syn), de l'additivité (Add) et de l'antagonisme (Ant) respectivement; NA = Non Applicable (**La méthode de Chou-Talalay est inutilisable lorsqu'une courbe effet- dose ne peut pas être générée ou est difficile à modéliser. Étant donné que la MON et la FB1 n'ont pas montré d'effet cytotoxique prononcé aux concentrations testées (leur faible solubilité ayant limité la détermination d'une courbe effet-dose complète), il n'a pas été possible d'établir une relation dose-réponse fiable).*

Les résultats obtenus ci-dessus rejoignent ceux précédemment observés *via* la méthode appliquée par Weber et al. (2005). En effet, quelle que soit l'approche utilisée, les effets cytotoxiques combinés des différents mélanges de mycotoxines étudiés sont principalement antagonistes. Cependant, les valeurs des IC_{BLISS} sont toujours légèrement inférieures à celles des $IC_{ADDITIVITE}$, indiquant une certaine pondération de l'antagonisme suggéré par les

IC_{ADDITIVITE}. En effet, plus la valeur de l'IC est élevée, plus l'antagonisme est important (Chou, 2006). Plus particulièrement, des IC supérieurs à 3,3 indiquent un antagonisme fort, alors que des IC inférieurs à 1,45 soulignent un antagonisme modéré (tableau 6). Ainsi, les IC_{BLISS} montrent de l'additivité $(0,9 \le IC \le 1,1)$ et/ou de l'antagonisme léger (IC $\le 1,2$) pour l'ensemble des mélanges à certaines concentrations, alors que seul de l'antagonisme (au moins) modéré (IC>1.2) est observé avec les IC_{ADDITIVITE}. Par ailleurs, bien que la méthode de Chou-Talalay indique majoritairement de l'antagonisme pour les mélanges DON+ZEA et NIV+T2, du synergisme fort (IC<0,3) est également observé à la plus forte concentration testée pour DON+ZEA, et à la plus faible pour NIV+T2. Quelques auteurs ont déjà utilisé plusieurs modèles simultanément pour caractériser les effets combinés des mycotoxines, et ont obtenu des conclusions différentes selon l'approche. C'est le cas, par exemple, de Tammer et al. (2007), qui ont observé du synergisme avec le modèle de Bliss et de l'antagonisme avec celui de Loewe sur la réduction de la production de cytokines INFs pour un mélange de quatre mycotoxines. Ces résultats soulignent donc un potentiel besoin de standardisation dans l'évaluation des effets combinés des mycotoxines.

Tableau 6 : Description de l'antagonisme et du synergisme dans les études de combinaisons
de mycotoxines analysées avec la méthode de l'indice de combinaison (IC) (Chou, 2006).

Gamme d'IC	Description de l'effet
<0,1	Synergisme très fort
0,1-0,3	Synergisme fort
0,3-0,7	Synergisme
0,7-0,85	Synergisme modéré
0,85-0,9	Synergisme léger
0,9-1,1	Additivité
1,1-1,2	Antagonisme léger
1,2-1,45	Antagonisme modéré
1,45-3,3	Antagonisme
3,3-10	Antagonisme fort
>10	Antagonisme très fort

Les points marquants de cette étude sont les suivants :

- Les mycotoxines DON, MON, FB1, ZEA, NIV et T2 réduisent la viabilité des monocytes THP-1 d'une manière dose-dépendante, T2 étant la plus cytotoxique.
- Les effets des différents mélanges testés sur la viabilité cellulaire semblent principalement antagonistes après 48h d'exposition, suggérant que la multi-exposition aux fusariotoxines peut être, dans les conditions testées, moins cytotoxique que l'exposition aux mycotoxines seules.
- La plupart des cellules viables ont perdu certains marqueurs de surfaces, tels que CD14 et CD71, après 48h d'exposition aux mycotoxines seules et en mélange, suggérant une potentielle perte de fonctionnalité de ces cellules ainsi qu'une possible perte de capacité de différenciation. Néanmoins, un effet plus important des mélanges que des toxines seules est observé sur la perte de ces marqueurs de surface. Ces résultats indiquent que la modification de l'état fonctionnel des cellules pourrait être davantage considérée comme un marqueur de cytotoxicité.
- Différents mécanismes de mortalité cellulaire sont observés entre les cellules exposées aux mycotoxines seules et celles exposées aux mélanges. Ces résultats suggèrent de possibles interactions ou une compétition entre les fusariotoxines lors de la co-exposition pour l'activation des voies de signalisation impliquées dans la mortalité des THP-1.
- A l'échelle moléculaire, la phosphorylation des MAPKs est observée après 1h d'exposition aux mycotoxines seules et en mélange à des doses faiblement cytotoxiques, ce qui souligne l'importante sensibilité des THP-1 et la rapidité de la réponse cellulaire suite à l'exposition aux fusariotoxines.

La figure 6 schématise de façon synthétique les principaux résultats obtenus dans cette étude.



Figure 6 : Représentation schématique des principaux effets biologiques observés dans l'étude n°1.



Figure 6 (suite): Représentation schématique des principaux effets biologiques observés dans l'étude n°1.
Partie II : Evaluation de l'hépatotoxicité aiguë des fusariotoxines et de la réponse cellulaire associée sur les cellules hépatiques humaines HepaRG

Deux articles sont associés à cette partie :

Etude n°2:

Hepatotoxicity of fusariotoxins, alone and in combination, towards the HepaRG human hepatocyte cell line

Food and Chemical Toxicology

Marie-Caroline Smith, Nolwenn Hymery, Samuel Troadec, Audrey Pawtowski, Emmanuel Coton, Stéphanie Madec

Etude n°3:

Differential impacts of individual and combined exposures of deoxynivalenol and zearalenone on the HepaRG human hepatic cell proteome

Journal of Proteomics

Marie-Caroline Smith, Emma Timmins-Schiffman, Monika Coton, Emmanuel Coton, Nolwenn Hymery, Brook Nunn, Stéphanie Madec

1. Effet des fusariotoxines seules et en mélange sur la mortalité et sur l'expression de certaines fonctions hépatiques des cellules HepaRG

Etude n°2:

Hepatotoxicity of fusariotoxins, alone and in combination, towards the HepaRG human hepatocyte cell line

Food and Chemical Toxicology (https://doi.org/10.1016/j.fct.2017.09.022)

Marie-Caroline Smith, Nolwenn Hymery, Samuel Troadec, Audrey Pawtowski, Emmanuel Coton, Stéphanie Madec

Dans cette deuxième partie, l'objectif était, cette fois, de mieux caractériser les effets de l'exposition et de la co-exposition aux fusariotoxines sur les cellules hépatiques humaines HepaRG proliférantes, le foie étant, comme indiqué précédemment, l'organe principal de la détoxification. Comme pour la première partie, les effets des fusariotoxines DON, MON, FB1, ZEA, NIV et T2 et des mélanges DON+MON, DON+FB1, DON+ZEA et NIV+T2 ont été évalués sur la viabilité et les mécanismes impliqués dans la mortalité cellulaire. La lignée cellulaire HepaRG est un modèle particulièrement adapté aux études de toxicité, puisqu'elle possède les performances métaboliques des hépatocytes primaires ainsi que les capacités de croissance des lignées transformées (Guillouzo et al., 2007; Nelson et al., 2017).

Pour permettre une comparaison appropriée des effets cytotoxiques individuels et combinés des différentes fusariotoxines sur les deux modèles cellulaires, THP-1 et HepaRG, les cellules hépatiques ont également été maintenues dans un état non différencié (pouvant ainsi être assimilées à des hépatoblastes). De plus, dans ce même souci de comparaison, les tests de cytotoxicité de cette étude ont été réalisés dans les mêmes conditions que dans la partie I. Ainsi, la toxicité de chaque fusariotoxine a d'abord été testée individuellement avec une large gamme de concentrations après 48h d'exposition *via* l'utilisation d'un test MTS, puis en mélange dans les mêmes conditions, en utilisant 5 concentrations induisant la même toxicité pour chacune des mycotoxines du mélange. Dans le contexte de cette étude, pour comparer

les toxicités individuelles et combinées des fusariotoxines, outre le modèle appliqué par Weber et al. (2005), nous avons utilisé deux autres approches, à savoir le « *Bliss Independence model* » (Bliss, 1939) et le « *combination index-isobolographic analyis* » (Chou, 2006; Chou and Talalay, 1984).

Dans un second temps, les mécanismes de mort cellulaire ont été étudiés après 3, 6, 12 et 18h d'exposition aux CI₅₀ (obtenues sur 48h), *via* un double marquage des cellules à l'annexine V-FITC/iodure de propidium suivie d'une analyse par cytométrie de flux.

Enfin, pour l'étude des effets des fusariotoxines sur les HepaRG à l'échelle moléculaire, nous avons choisi de nous focaliser sur le mélange DON+ZEA, qui est l'un des mélanges de fusariotoxines les plus répandus dans le monde. Plus particulièrement, les niveaux d'expression des gènes codant pour certaines fonctions spécifiques des cellules hépatiques ont été mesurés après 1h d'exposition au DON, à la ZEA et au mélange DON+ZEA à des concentrations cytotoxiques très faibles (CI10 obtenues après 48h). Nous avons choisi d'étudier des gènes codant pour deux enzymes importantes impliquées dans la Phase I du métabolisme de détoxification des hépatocytes : CYP3A4 et CYP4F3B (appartenant à la famille CYP450), deux facteurs de transcription : C/EBP α et HNF4 α , ainsi que trois protéines spécifiques du foie : la transferrine et l'albumine, qui sont des protéines du plasma, et l'aldolase B, qui est une enzyme du métabolisme du glucose. Une diminution de l'expression des gènes codant pour l'un de ces facteurs pourrait mettre en évidence une perte d'une des fonctions hépatiques spécialisées. En revanche, une augmentation de leur expression suggérerait une « hyperactivité » cellulaire pouvant conduire, par exemple, à un métabolisme plus rapide des xénobiotiques. De plus, dans le but d'observer de potentielles corrélations entre l'expression des gènes et celles des protéines, nous avons également mesuré l'expression de certains de ces facteurs par Western Blot, à savoir le cytochrome CYP3A4 et l'albumine, dans les mêmes conditions d'exposition que précédemment. De plus, l'expression de la claudine-1 (protéine des jonctions serrées) a également été mesurée en tant qu'indicateur de la perméabilité de la monocouche cellulaire.

La figure 7 schématise de façon synthétique les principaux effets biologiques évalués dans cette étude.



Figure 7 : Résumé schématique des différents effets biologiques et conditions évalués dans l'étude n°2.

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Hepatotoxicity of fusariotoxins, alone and in combination, towards the HepaRG human hepatocyte cell line



Food and Chemical Toxicology

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ABSTRACT

While the reality of mycotoxin co-occurrence in food commodities is now established, their effects in mixtures are not well studied. The present study investigated the individual and combined effects of deoxynivalenol (DON), nivalenol (NIV), T-2 toxin (T2), fumonisin B1 (FB1), zearalenone (ZEA) and moniliformin (MON) fusariotoxins on cell viability and cell death mechanisms in proliferating HepaRG cells, a human derived liver cell line. In addition, DON-ZEA being one of the most widespread mycotoxin mixtures in grains worldwide, its effect on the expression levels of genes encoding for sets of hepatocyte-specific functions was studied. After 48 h, T2 appeared to be the most cytotoxic tested fusariotoxins, followed by NIV, DON and ZEA. Furthermore, at low cytotoxic doses, all tested fusariotoxin mixtures (DON-MON, DON-FB1, DON-ZEA and NIV-T2) acted synergistically on cell death. Interestingly, during the first 18 h of exposure, only FB1 and ZEA alone and in combination with DON seemed to induce cell apoptosis and necrosis. At the gene level, after only 1 h, DON-ZEA combination induced expression of drug-metabolizing enzymes contrary to individual exposures. Thus, the observed synergy of fusariotoxin mixtures suggested that their simultaneous presence in food commodities can induce a toxic risk that should be better taken into consideration.

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1. Introduction

Deoxynivalenol (DON), nivalenol (NIV), T-2 toxin (T2), fumonisin B1 (FB1), zearalenone (ZEA) and moniliformin (MON) mycotoxins are secondary metabolites produced by some *Fusarium* species, and usually called fusariotoxins. These compounds are mainly secreted, in an environment-dependent manner, on cereal crops before or immediately after harvest (Bryden, 2012). In addition, they can contaminate finished processed food products due to their resistance to most food and feed processing step and treatments (Bullerman and Bianchini, 2007). Fusariotoxins are very widespread contaminants naturally found in food commodities, mainly in the North Temperate Zone (Smith et al., 2016). Nowadays, their implication in many human and animal health disorders is well-established (da Rocha et al., 2014; Richard, 2007). Their adverse health effects are various, depending on numerous factors such as exposure time, doses and mycotoxin combinations.

Trichothecenes (TCT) are the most important fusariotoxin group, and DON, NIV and T2 being among the most frequent ones. At the molecular level, TCT appear to display multiple inhibitory effects on primary metabolism of eukaryotic cells, including inhibition of DNA, RNA and protein synthesis as well as mitochondrial function (da Rocha et al., 2014). Thus, they are especially toxic to organs containing actively dividing cells (Kongkapan et al., 2016). Another relevant fusariotoxin family corresponds to fumonisins (FUM), which are the most widely distributed Fusarium toxins with DON, and are found in more than 60% of cereal samples analyzed worldwide by Rodrigues and Naehrer (2012). The most abundantly produced molecule of the FUM family is fumonisin B1 (FB1). At the molecular level, FUM inhibit the ceramide synthase enzyme which is responsible for sphinganine and sphingosine acylation. Sphinganine accumulation in tissues initiates a cascade of events that may cause toxicity, especially at the liver and kidney levels, as well as carcinogenicity (Bosco and Molle, 2012; Bryden, 2012; Richard, 2007). Regarding ZEA, although this fusariotoxin is usually nonlethal to animals, it is very widespread in cereals worldwide,

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Abbreviations					
CI	Combination Index				
DON	Deoxynivalenol				
FB1	Fumonisin B1				
FUM	Fumonisins				
IC	Inhibitory concentration				
IJA	Independent Joint Action				
MON	Moniliformin				
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-				
	carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-				
	tetrazolium)				
NIV	Nivalenol				
PI	Propidium iodide				
PS	Phosphatidylserine				
RA	Response Additivity				
RT-qPCR	Real-time quantitative polymerase chain reaction				
T-2	T-2 toxin				
TCT	Trichothecenes				
ZEA	Zearalenone				

found in about 45% of cereal samples analyzed by Rodrigues and Naehrer (2012), and it is therefore of interest from the safety and economic points of view. This non-steroidal estrogenic mycotoxin triggers estrogen gene activation and causes functional and morphological alterations on reproductive organs (Tatay et al., 2014; Zinedine et al., 2006). Moreover, ZEA is mainly metabolized in the liver, which seems to be an important target too. Indeed, ZEA was found to be hepatotoxic by inducing adverse liver lesions with subsequent development of hepatocarcinoma (Hassen et al., 2007; Wang et al., 2014). Beyond these major fusariotoxins, the members of the Fusarium genus can produce other lesser-studied toxins, called emerging mycotoxins, such as MON (Jestoi, 2008). They are considered less toxic than major fusariotoxins but have a high occurrence in food products, sometimes even in high concentrations (Escrivá et al., 2015). The toxicological effects of MON are currently poorly documented. Nevertheless, at the cellular level, MON is known to inhibit mitochondrial pyruvate and α-ketoglutarate oxidations (Cetin and Bullerman, 2005).

Among the infinite number of possible mycotoxin mixtures found in food and feed commodities, combinations of fusariotoxins are particularly widespread, mostly in the North Temperate Zone. More particularly, DON in combination with ZEA is the most encountered mycotoxin mixture in Europe and North America, and their production by similar fungi species can explain their cooccurrence in agricultural products. In addition, DON is often found to co-occur with FUM or MON mainly in maize. Furthermore, because several fungi species can produce NIV and T2 mycotoxins simultaneously or in a quick succession, the NIV-T2 mixture is also commonly found in grains and is therefore relevant (Smith et al., 2016).

Noteworthy, alteration of liver functions is one of the most commonly described effects of acute mycotoxin exposure (Pitt, 2000). Indeed, the liver being the main detoxification organ, hepatocytes represent one of the primary targets of these toxins, and are therefore a relevant *in vitro* toxicology standard model. Since more than 50% of the drugs that induce liver injury in human clinical trials are not hepatotoxic to animals, human hepatocytes are needed for more accurate *in vitro* screening of drug toxicity (Olson et al., 2000). In particular, the HepaRG cell line, derived from a human hepatocellular carcinoma, seems to be a surrogate to

primary human hepatocytes for toxicity studies, as this cell line possesses both the primary hepatocyte metabolic performances and hepatic cell line growth capacity (Guillouzo et al., 2007; Nelson et al., 2017).

While fusariotoxins are generally present simultaneously in food and feed, their combined effects are still little studied. Yet, their possible interactions can potentially lead to either antagonistic, additive or synergistic effects (Smith et al., 2016). The aim of this study was to compare the single and combined effects of various fusariotoxins on the HepaRG human liver cell line and then to focus on DON-ZEA, one of the most encountered mycotoxin mixtures in grains (Smith et al., 2016) to study the expression levels of genes encoding for a set of liver-specific functions.

2. Materials and methods

2.1. Mycotoxins

Mycotoxin standards, namely DON (CAS#51481-10-8) with purity >98%, MON (CAS#71376-34-6) with purity >98% and produced from *Fusarium proliferatum*, FB1 (CAS#116355-83-0) with purity >98% and produced from *Fusarium moniliforme*, ZEA (CAS#17924-92-4) with purity >99% as well as T2 (CAS#21259-20-1) with purity >98% and produced from *Fusarium* sp., were purchased from Sigma-Aldrich (St. Louis, MO, USA). NIV standard (CAS#23282-20-4) with purity >99% was obtained from Oskar-Tropitzsh e.K. (Marktredwitz, Germany). All standards were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20 °C.

2.2. Cell and culture conditions

Human hepatocytes (HepaRG) were acquired from Biopredic International (Saint Grégoire, France). They were cultured in William's E medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin and 50 µM hydrocortisone hemisuccinate at 37 °C with 100% relative humidity (RH) in a 5% CO₂ atmosphere. Culture medium was renewed every 2–3 days with fresh growth medium. The cells were passaged every 2 weeks at a density of 2.7×10^4 cells/cm² by a short time exposure (<5 min) with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Sigma-Aldrich, St. Louis, MO, USA), and reseeded in a fresh medium.

2.3. Cytotoxicity evaluation by MTS assay

To explore the individual and combined cytotoxic effects of DON, MON, FB1, ZEA, NIV and T2 on undifferentiated HepaRG cells, 2.5×10^5 cells were treated with increasing doses of these fusariotoxins added to the culture medium (from 10^{-9} M to 10^{-5} M for DON, MON, FB1 and NIV, and from 10^{-9} M to 10^{-4} M for T2 and ZEA), followed by incubation at 37 °C in 24-well-plates during 48 h. The final concentration of solvent (DMSO) in cell culture containing mycotoxins did not exceed 2%. Appropriate control cultures without mycotoxin but containing the same amount of solvent were included as negative controls. Concerning the mycotoxin mixtures, a fixed ratio was chosen to facilitate the concentrationresponse analysis of each mycotoxin mixture. More specifically, the tested binary combination ratios were 1/7.5 for DON-ZEA and 1/ 0.067 for NIV-T2. These ratios were based on the relative toxicity of the two mycotoxins from each mixture, and were calculated from these mycotoxin respective IC₅₀ values (concentrations inhibiting 50% of cell viability from the negative control). Thus, the selected DON concentrations (namely 0.2, 2, 6, 8 and $10 \mu M$) were multiplied

by 7.5 to obtain ZEA doses, while NIV concentrations (namely 0.6, 2, 3, 4 and 10 μ M) were divided by 15 to obtain T2 doses (based on IC₅₀ ratio calculations). Regarding MON and FB1, 10 μ M (corresponding to their dissolution limit in DMSO) were used in combination with all selected DON doses. Inhibitory concentration values were obtained from preliminary individual cytotoxicity experiments using the theoretical dose-response curves established with Microsoft TableCurve 2D Software v.5.01 (Microsoft, Redmond, WA, USA). Dose-response curves were obtained by plotting the percentage of cell viability against the log of mycotoxin concentrations.

Mycotoxin cytotoxicity towards HepaRG lineage cells was evaluated using the Promega CellTiter 96AQueous Non-Radioactive cell proliferation assay (Promega, Madison, WI). This colorimetric method determines mitochondrial activity based on the reduction of a tetrazolium salt (MTS 3-[4,5-dimethylthiazol-2-yl]-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay) by mitochondrial dehydrogenases in viable cells. At the end of the incubation in the presence of mycotoxins, alone or in combination, under normal incubation conditions, the culture medium was removed and cells were washed with PBS. Then, they were harvested by a short exposure time (<5 min) to 100 µL of trypsin-EDTA. To stop EDTA-trypsin action, 400 µL of FBS were added in each well and the resulting volume was transferred in a tube prior to centrifugation (10 min, 400 rpm). Supernatant was removed and cells were transferred in 96-well-plates. Then, 100 µL PBS were added in each well as well as 20 μ L CellTiter 96AQ_{ueous} Non-Radioactive solution and the cells were further incubated for 3 h. Formazan (MTS metabolite) was quantified by reading the absorbance at 490 nm on a Multiskan FC plate reader (Thermo Scientific. Madison, WI). Three independent experiments were performed for each condition and cell viability obtained for the negative control was defined as 100%.

2.4. Assessment of cell mortality mechanisms by flow cytometry

To investigate early cell mortality mechanisms induced by mycotoxin single and combined exposures on HepaRG cell cultures, 5×10^5 undifferentiated confluent cells seeded in 12-well-plates were treated for 3, 6, 12 and 18 h with DON, ZEA, NIV and T2 at their respective IC₅₀ (namely 7.35 μ M, 55.1 μ M, 2.84 μ M, and 0.19 μ M) or at solubility limits for MON and FB1 (10 µM). These same concentrations were used in combination. The measurement of viable, apoptotic and necrotic cell number was performed by annexin V-FITC/propidium iodide (PI) cells double staining (Miltenyi Biotec, Bergisch Gladbach, Germany). Following the manufacturer's instructions, at the end of the incubation time, cell cultures were harvested, washed and suspended in 100 μ L 1 \times binding buffer followed by an addition of 10 µL of annexin V-FITC. After homogenization, the cells were incubated in the dark at room temperature for 15 min, and then washed in $1 \times$ binding buffer. Finally, cell pellets were resuspended in 500 μ L of 1 \times binding buffer and 5 μ L of PI were added. Flow cytometry analysis was performed using a BD Accuri C6 flow cytometer associated with the BD Accuri C6 software (BD Biosciences). FITC-fluorescence (FL-1) and PI fluorescence (FL-3) were collected through a 530 nm bandpass filter and a >670 nm bandpass filter respectively. Compensation for spectral overlap between FL-1 and FL-3 channels was performed using unlabeled and single stained cell populations. Annexin V positive and PI negative cells were considered to be apoptotic cells in the early stage, whereas Annexin V and PI positive cells were considered to be necrotic or apoptotic cells in the late stage. Three independent experiments were performed for each condition and 10,000 events were collected from each sample.

2.5. RT-qPCR for gene expression assessment

For the evaluation of hepatocyte-specific factor expression (namely CYP4F3B, CYP3A4, C/EBPa, HNF4a, aldolase B, transferrin and albumin), 3 \times 10⁶ undifferentiated confluent HepaRG cells seeded in 25 cm²-flasks were exposed to DON and ZEA, alone and in combination, at their IC₁₀ (namely 0.21 μ M and 20 μ M respectively) for 1 h. The final concentration of solvent (DMSO) in cell culture containing mycotoxins did not exceed 0.5%. Appropriate control cultures without mycotoxin but containing the same amount of solvent were included as negative controls. At the end of the incubation period, the culture medium was removed and the cells were washed with PBS. Then, the cells were harvested and centrifuged. The dry cell pellets were stored at -80 °C until RNA extraction.

Total RNA was extracted from dried cell pellets using the RNeasy Protect Mini Kit from Qiagen (Valencia, Canada). RNA quantity and quality were assessed with a NanoDrop 1000 spectrophotometer from Thermo Scientific. For cDNA synthesis, 1 μ g of total RNA was reverse transcribed for each sample using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystem). Reverse transcription proceeded for 10 min at 25 °C, 2 h at 37 °C and 5 min at 85 °C using a peqSTAR 2× thermal cycler (PEQLAB – Life Science, VWR, Erlangen, Germany). The obtained cDNAs were then stored at -80 °C.

Quantitative polymerase chain reaction (qPCR) was performed by real-time fluorescent PCR using a C1000 thermal cycler with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). PCR primers designed by Madec et al. (2011) corresponding to *TBP* (housekeeping gene), Hs00427620_m1; CYP3A4, Hs00604506_m1; C/EBPα, Hs00269972_s1; $HNF4\alpha$, Hs00230853_m1; aldolase B, Hs01551887_m1; transferrin, Hs1067777_m1 and albumin, Hs00609477_m1 genes were purchased from Life Technologies Ltd, Thermo Fisher Scientific (Paisley, UK) and used with the $5 \times$ HOT FIREPol Probe gPCR Mix Plus (Solis BioDyne, Tartu, Estonia) for gPCR amplification. GAPDH (Forward-CCCATCACCATCTTCCAGGAG; primer: **Reverse-primer:** GTTGTCATGGATGACCTTGGC) (housekeeping gene) and CYP4F3B (Madec et al., 2011) genes were synthesized by Invitrogen, Thermo Fisher Scientific (Carlsbad, CA, USA) and used with the $5\times$ HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). For each transcript, a standard curve was constructed using the purified PCR product generated for each specific primer pair. Single reactions were prepared for each cDNA along with each series of dilution using the DNA-binding dye 5× HOT FIREPol Probe qPCR Mix Plus or the 5× HOT FIREPol EvaGreen qPCR Mix Plus from Solis BioDyne according to the manufacturer's recommendations. The cycling conditions for the $5 \times$ HOT FIREPol Probe qPCR Mix Plus were 1 cycle of activation at 95°C/15min, followed by 40 three-segment cycles of denaturation at 95°C/15 s and annealing/elongation at 60° C/60 s. Concerning the 5× HOT FIREPol EvaGreen qPCR Mix Plus, the cycling conditions were 1 cycle of activation at 95°C/ 15min, followed by 40 three-segment cycles of denaturation at 95°C/15 s, annealing at 62°C/20 s and elongation at 72°C/20 s. The baseline adjustment method of the Bio-Rad CFX Manager 2.1 software was used to determine the Ct in each reaction. All samples were amplified in triplicates and the mean value was used for further analysis. Relative gene expression levels were normalized to GAPDH or TBP gene expression, and results were represented as fold change relative to the level expressed in untreated control cells.

2.6. Western blot for protein expression measurement

Albumin, CYP3A4 and claudin-1 expression was assessed by Western blot following the same exposure conditions as in § 2.5. Then, cell pellets from HepaRG cell cultures were lysed with 100 µL of ice-cold cell lysis buffer (NP40 Cell Lysis Buffer, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1 mM PMSF and a protease inhibitor cocktail tablet (Complete EDTA-free, Roche Diagnostics, Mannheim, Germany). After 30 min on ice with vortexing at 10 min intervals, cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. The clear lysates were transferred in clean microfuge tubes and protein concentrations were determined using the colorimetric Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). For each sample, 20 µg of total protein were denatured by boiling at 99 °C for 10 min with a 1:4 dilution of $4\times$ Laemmli sample buffer (0.06 M Tris-HCl pH 6.8, 20% v/v SDS 10%, 10% v/v glycerol, 5% v/v bromophenol blue and 5% v/v β-mercaptoethanol in distilled water), completed with distilled water to 20 µl. Each sample was entirely loaded onto a 12% polyacrylamide gel for protein detection and ran at 80 mA (Bio-Rad Mini-PROTEAN 3 Cell Electrophoresis System, Bio-Rad Laboratories, Hercules, CA, USA). Then, following the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, US), a transfer onto nitrocellulose (0.45 µm, GE Healthcare-Amersham, Buckinghamshire, UK) was conducted at 250 mA for 1 h. Membranes were saturated during 1 h with TBS (Tris-buffered saline)-0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) washing buffer containing 5% w/v nonfat dry milk. Membranes were probed overnight at 4 °C with appropriate primary antibody diluted in TBS-0.1% Tween 20 containing 5% BSA or nonfat dry milk (1:1000). Anti-active polyclonal CYP3A4, albumin, claudin-1 and β -actin (housekeeping protein) antibodies were purchased from Cell Signaling (Danvers, MA, USA) and stored at -20 °C. A horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in TBS-0.1% Tween 20 and 5% nonfat dry milk) (Cell Signaling) was used for incubating membranes during 1 h. Band detection was performed with ECL Prime Western Blotting Detection Reagent (GE Healthcare-Amersham, Buckinghamshire, UK) and membranes were scanned using the G-Box (Syngene, Cambridge, UK). Five independent experiments were performed and blots were analyzed using the Gene Tools analysis software (Syngene, Cambridge, UK). The relative protein expression levels were normalized to β -actin expression and the results were represented as fold change relative to the level expressed in untreated control cells.

2.7. Mycotoxin combinatory effect analysis

To evaluate the mycotoxin combined effects, three different conceptual models were applied: i) the response additivity (RA) (Foucquier and Guedj, 2015), also defined as the arithmetic definition of additivity (Alassane-Kpembi et al., 2016) or linear interaction effect (Slinker, 1998), ii) the Bliss independence model (Bliss, 1939), also referred to as response addition (Alassane-Kpembi et al., 2016) or independent joint action (IJA) (Vejdovszky et al., 2016), and iii) the median effect principle of the mass action law (Chou, 2006), known as Chou-Talalay method or combination indexisobologram analysis (Chou, 2006; Chou and Talalay, 1984). According to Foucquier and Guedj (2015), these approaches may be divided into effect-based and dose-effect-based approaches. Methods following an effect-based strategy (i.e. RA and IJA) compare the effect resulting from the combination of two toxins directly to the effects of its individual components, while those following a dose-effect-based strategy (i.e. combination indexisobologram analysis) compare different agents having nonlinear dose-effect curves by finding what amount or concentration of each produces the same quantitative effect. All these models allow characterizing mycotoxin interactions in a mixture, but do not elucidate the mechanisms supporting the observed interactions.

For each model, we provided a combination index (CI) -

recognized as a standard measure of combination effect – that indicates a higher (CI < 0.9), lower (CI > 1.1) or similar (0.9 \leq CI \leq 1.1) effect than the expected additive effect, for all mycotoxin combinations and all tested concentration combinations.

2.7.1. Response additivity

In the RA approach, the expected toxic effect of a mixture is defined as the arithmetic sum of the observed toxic effects for individual compounds when tested separately. Thus, the expected cytotoxic effect of a binary mycotoxin mixture is calculated as follows:

Expected cytotoxic effect (mycotoxin 1 + mycotoxin 2) = observed cytotoxic effect (mycotoxin 1) + observed cytotoxic effect (mycotoxin 2).

When the measured cytotoxicity values are significantly above or below the expected values, the results are considered as synergistic or antagonistic respectively, while no significant differences suggest additivity.

The CI can be calculated as follows:

 $CI_{RA} = [observed effect (mycotoxin 1) + observed effect (mycotoxin 2)]/observed effect (mycotoxin 1 + mycotoxin 2).$

2.7.2. Independent joint action

The Bliss independence model (Bliss, 1939) assumes that toxins act independently from one another in such a manner that neither of them interferes with the other. The expected combination effect can be expressed as a probability (ranging within 0 and 1) by the following equation:

Expected cytotoxic effect (mycotoxin 1 + mycotoxin 2) = observed cytotoxic effect (mycotoxin 1) + observed cytotoxic effect (mycotoxin 2) - observed cytotoxic effect (mycotoxin 1) × observed cytotoxic effect (mycotoxin 2).

As for the RA strategy, the expected additive values are compared to observed values, and interactions between compounds are indicated when the combinatory effect is not additive.

The CI can be calculated as follows:

 $Cl_{IJA} = [observed effect (mycotoxin 1) + observed effect (mycotoxin 2) - observed effect (mycotoxin 1) × observed effect (mycotoxin 2)]/observed effect (mycotoxin 1 + mycotoxin 2).$

2.7.3. Chou-Talalay method

The Chou-Talalay model, derived from median-effect principle of the mass action law, allows modeling the individual dose-effect curves and the additive response that could be expected from the combined effect of several drugs (Chou, 2006; Chou and Talalay, 1984). Parameters as the median-effect dose (i.e. IC₅₀), fraction affected by concentration and coefficient signifying the shape of the dose-effect relationship are relevant in the equation. For all binary mycotoxin combinations, CI values were generated with CompuSyn software version 3.0.1 (ComboSyn, Inc., Paramus, NJ. 07652 USA).

This method is unusable when a dose-effect curve is not available or difficult to model (Foucquier and Guedj, 2015). Since MON and FB1 did not show a pronounced cytotoxic effect in the concentration range tests, it was not possible to establish a reliable dose—response relation. Their low solubility limited the determination of a complete dose-response curve exceeding 10% effect. Thus, only the models of RA and IJA were used to characterize the combined effects of the DON-MON and DON-FB1 mixtures.

2.8. Statistical analysis

For MTS assays, the cell viability percentage mean of three independent experiments \pm standard error of mean (SEM) were used for statistical analyses, which were performed using the unpaired Student's t-test for comparison with control. Values of P < 0.05 were considered as significant.

For annexin V-FITC/PI double staining assay, the mean of viable, apoptotic and necrotic cell populations of three independent experiments \pm SEM were used for statistical analyses, which were performed using Statistica for Windows (version 10; StatSoft). After verifying normal data distribution and variance homogeneities, different mean value groups were compared to control values and to other exposure time according to the least significant differences (LSDs) test of multifactor ANOVA analysis followed by HSD Turkey's post hoc test (P-values < 0.05).

Regarding RT-qPCR and Western blot analyses, the data were expressed as the mean of 3 or 5 independent experiments \pm SEM, respectively. Statistical analyses were performed using the unpaired Student's t-test for comparison with control (P-values < 0.05).

3. Results

3.1. Effects of mycotoxin single and combined exposures on cell viability

After 48 h of mycotoxin single exposure, only DON, ZEA, NIV and T2 decreased HepaRG cells viability in a dose-dependent manner (Fig. 1). More particularly, these four fusariotoxins exerted clear toxicity at concentrations above 0.1 μ M for T2, 1 μ M for DON and NIV, and 10 μ M for ZEA (Fig. 1F, 1A, 1E and 1D respectively). Thus, T2 showed the highest cytotoxicity by inducing 100% cell mortality at 1 μ M, followed by NIV and DON, inducing about 100% and 80% cell mortality respectively at the highest tested concentration (i.e. 10 μ M). Concerning MON, FB1 and ZEA, at 10 μ M (solubility limit in DMSO for MON and FB1), only 10% of cell mortality was observed (Fig. 1B, 1C and 1D respectively). However, at 100 μ M, ZEA induced more than 90% of cell mortality. The determined IC₁₀, IC₃₀ and IC₅₀ values for each mycotoxin were reported in Table 1.

For each tested mycotoxin mixture (DON-MON, DON-FB1, DON-ZEA and NIV-T2), five concentration combinations were tested (Fig. 2). By analyzing the CI values calculated using different approaches (Table 2), and obtained from 48 h co-exposure experiments, synergistic effects on cell viability reduction were mainly observed after DON-MON and DON-FB1 co-exposures. For these mixtures, additivity was only observed at the highest tested concentrations (namely 10 μ M DON + 10 μ M of either MON or FB1) with both RA and IJA models. In the same way, exposure of HepaRG cells to the DON-ZEA mixture mainly led to synergism, as shown by the three models. Only the two highest concentration combinations (namely 8 μM DON + 60 μM ZEA and 10 μM DON + 75 μM ZEA) led to antagonism according to the RA approach and only the latter combination led to additivity with the IJA prediction. Regarding the NIV-T2 mixture, synergism, additivity and antagonism were observed depending on the tested concentrations and the applied model. More specifically, the lowest tested concentration combination led to synergistic effects with the three models, whereas the two following dose combinations led to antagonism. At the two highest combinations, antagonistic, additive and synergistic effects on cell viability reduction were obtained according to the RA, IJA and CI-isobologram approaches respectively.

For the DON-ZEA and NIV-T2 mixtures, the evolution of CI values for graded levels of cytotoxicity is presented in Fig. 3. Along the graded cytotoxicity levels, the DON-ZEA combination showed synergism, with the highest limit of the 95% confidence interval for the CI values lower than 0.5 (Fig. 3A). By contrast, the NIV-T2 mixture mainly led to antagonism and additivity (Fig. 3B). More particularly, low doses were antagonistic, medium doses behaved additively and only very high concentrations led to a slight

synergism.

3.2. Cell mortality mechanisms involved after mycotoxin single and combined exposures

After mycotoxin mono and co-exposures on HepaRG cells at their respective IC₅₀ or solubility limits, quantitative variations of apoptotic and necrotic cells were followed for 3, 6, 12 and 18 h using the annexin V-FITC/PI cell double staining assay (Fig. 4). The results after 3 h of single and combined mycotoxin exposures showed that only the DON-FB1 mixture significantly reduced cell viability compared to the control condition (about 27% cell mortality vs 17% for control) by inducing early cell apoptosis. Indeed, the percentage of apoptotic cells among the HepaRG population exposed to DON-FB1 (about 20%) was significantly higher than for control (about 12.5%) (P < 0.05). Regarding the results from 6 h of exposure, no mycotoxin or mixture seemed to significantly reduce cell viability. After 12 h of exposure, cell viability was significantly reduced by DON-ZEA mixture only (about 29% cell mortality), by inducing early cell apoptosis and necrosis simultaneously. Herein, the percentage of apoptotic and necrotic cells (about 21% and 8.5% respectively) was significantly higher than for control (12.5% and 4.2% respectively) (P < 0.05). Finally, after 18 h of exposure, the results showed that FB1 and ZEA, alone and in combination with DON, reduced significantly HepaRG viability (about 28% for FB1 and DON-FB1 and 34% for ZEA and DON-ZEA) by inducing both early cell apoptosis and necrosis. Indeed, the percentage of apoptotic and necrotic cells (20% and 8% for FB1 and DON-FB1, respectively and 25% and 9% for ZEA and DON-ZEA. respectively) was significantly higher than for the control condition (P < 0.05).

3.3. Effect of mycotoxin single and combined exposures on hepatocyte functions

Expression levels of genes encoding for a set of liver-specific functions were measured by RT-gPCR from HepaRG cells exposed for 1 h with DON and/or ZEA, and the results were reported in Table 3. Among the expressed liver functions, we focused on liverspecific functions (transferrin and albumin), glycolytic enzymes (aldolase B), detoxification enzymes (CYP3A4) and liver-specific transcription factors (HNF4a) as well as specific hepatocytes differentiation markers (CYP4F3B drug-metabolizing enzyme and C/ EBPa transcription factor). Single exposure experiments showed that DON significantly reduced the expression of genes encoding for the transcription factor C/EBPa gene as well as the plasma protein transferrin, while it significantly increased the expression of the albumin encoding gene. After ZEA individual exposure, only the expression of the transferrin encoding gene was downregulated. Interestingly, the DON-ZEA mixture showed higher gene expression changes compared to mycotoxin single exposures. Indeed, the combination induced an up-regulation of all the studied liver-specific factor genes, with the exception of the HNF4 α and C/ EBPα transcription factors.

The expression of albumin, CYP3A4 and claudin-1 at the protein level was then assessed by Western blot. As for RT-qPCR experiments, the observed individual protein expression in the control conditions was arbitrarily set to 1 and results were then expressed as fold of control (Fig. 5). Individual mycotoxin exposures showed that DON only reduced significantly albumin expression (about 2.1 fold) while ZEA did not induce any expression changes for the studied proteins compared to the control condition. Regarding the DON-ZEA combination, the results showed that the expression of the drug-metabolizing enzyme CYP3A4 was significantly enhanced compared to control (about 3.1 fold) and, on the contrary, the expression of the tight-junction protein claudin-1 was reduced

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Fig. 1. Effects of (A) DON, (B) MON, (C) FB1, (D) ZEA, (E) NIV and (F) T2 on HepaRG cells after 48 h exposure (mean percentage \pm SEM of cell viability quantified using MTS bioassay, N = 3) * = cell viability measured mean significantly different from negative control (100%) (P < 0.05).

Concentrations of DON, MON, FB1, NIV, T2 and ZEA (μ M) resulting in 10, 30 and 50% inhibition of HepaRG viability (IC₁₀, IC₃₀ and IC₅₀, respectively) after 48 h exposure (calculated using TableCurve 2D software).

Mycotoxin	IC ₁₀ (μM)	IC ₃₀ (μM)	IC ₅₀ (μM)
DON	0.20 ± 0.04	2.34 ± 0.31	7.35 ± 0.33
MON	>10 ^a	>10 ^a	>10 ^a
FB1	>10 ^a	>10 ^a	>10 ^a
ZEA	20.01 ± 1.30	33.14 ± 0.77	55.12 ± 2.71
NIV	0.82 ± 0.27	2.25 ± 0.11	2.84 ± 0.05
T2	0.01 ± 0.00	0.07 ± 0.01	0.19 ± 0.02

^a Highest tested concentration.

(about 1.7 fold). Concerning albumin, no significant difference with the control was observed.

4. Discussion

Due to the shift from single analyte methods to multi-target methods over the last years, there has been increasing evidence that humans and animals may be exposed to several mycotoxins

simultaneously through food consumption (Streit et al., 2013; Varga et al., 2013). Moreover, a cocktail of mycotoxins can lead to a possible higher risk of adverse effects than the intake of a single mycotoxin since their possible interactions can not only lead to additive effects but also potentially to synergistic ones (Smith et al., 2016). Noteworthy, antagonistic effects are also possible. However, the assessment of the toxicological impact of mycotoxin mixtures is still rare and studies examining the mechanistic cellular response to mycotoxins (alone or in mixture) are scarse. Because of their occurrence and toxicity, some fusariotoxins such as DON, FUM and ZEA are the focus of legal regulations or guidance in many countries. Nevertheless, despite their co-occurrence with these fusariotoxins, NIV, T2 and MON are not yet regulated, even by the European Commission which has one of the most stringent regulations in the world, with numerous mycotoxins and commodities concerned as well as more restrictive levels. In addition, to our best knowledge, as in the rest of the world, to this date the European Commission does not consider the combined toxicological effects of mycotoxins (Smith et al., 2016).

The present study was designed to compare the single and combined effects of various fusariotoxins on the HepaRG human



Fig. 2. Observed (mean ± SEM of the fraction of cell viability affected and quantified using MTS bioassay, N = 3) and predicted (using the Response Additivity (RA) and Independent Joint Action (IJA) models) cytotoxic effects of (A) DON-MON, (B) DON-FB1, (C) DON-ZEA and (D) NIV-T2 co-exposure on HepaRG cell viability after 48 h exposure.

Combination Index (CI) values (as function of concentration combination) of the DON-MON, DON-FB1, DON-ZEA and NIV-T2 binary mixtures on HepaRG cells after 48 h exposure, calculated according to the Response Additivity (CI_{RA}), Independent Joint Action (CI_{IJA}) and CI-isobologram models.

Mycotoxin mixture		CI _{RA}	CI _{IJA}	CI- isobologram
DON-MON	0.2 μM DON +10 μM MON	0.51 Syn	0.49 Syn	NA
	$2 \ \mu M \ DON + 10 \ \mu M \ MON$	079 Syn	0.73 Syn	
	$6 \ \mu M \ DON + 10 \ \mu M \ MON$	0.62 Syn	0.57 Syn	
	8 μM DON +10 μM MON	0.80 Syn	0.73 Syn	
	10 μM DON +10 μM MON	1.01 Add	0.92 Add	
DON-FB1	0.2 μM DON +10 μM FB1	0.42 Syn	0.40 Syn	NA
	2 µM DON +10 µM FB1	0.69 Syn	0.64 Syn	
	6 μM DON +10 μM FB1	0.59 Syn	0.55 Syn	
	8 µM DON +10 µM FB1	0.78 Syn	0.72 Syn	
	10 µM DON +10 µM FB1	0.95 Add	0.87 Syn	
DON-ZEA	0.2 μM DON +1.5 μM ZEA	0.75 Syn	0.72 Syn	0.32 Syn
	$2 \ \mu M \ DON + 15 \ \mu M \ ZEA$	0.70 Syn	0.62 Syn	0.43 Syn
	$6 \ \mu M \ DON + 45 \ \mu M \ ZEA$	0.89 Syn	0.71 Syn	0.20 Syn
	8 μM DON +60 μM ZEA	1.18 Ant	0.86 Syn	0.20 Syn
	10 μ M DON +75 μ M ZEA	1.40 Ant	0.93 Add	0.14 Syn
NIV-T2	0.6 μM NIV +0.04 μM T2	0.80 Syn	0.75 Syn	0.48 Syn
	2 μM NIV +0.13 μM T2	1.34 Ant	1.08 Add	1.55 Ant
	3 μM NIV +0.2 μM T2	1.83 Ant	1.34 Ant	2.28 Ant
	4 μM NIV +0.27 μM T2	1.35 Ant	0.93 Add	0.46 Syn
	10 μ M NIV +0.67 μ M T2	1.74 Ant	1.01 Add	0.49 Syn

 $CI < 1, 0.9 \le CI \le 1.1$ and CI > 1.1 indicate synergism (Syn), additivity (Add) and antagonism (Ant), respectively; NA = Non Applicable.

liver cell line and then to focus on DON-ZEA, one of the most encountered mycotoxin mixtures in grains, for the mechanistic aspects. For the toxicological aspects, several parameters were studied, including cell viability, apoptosis and necrosis. Concerning the mechanistic aspects of the cellular response to mycotoxins, the expression levels of 7 genes encoding for a set of liver-specific functions in HepaRG cells and of targeted hepatocyte proteins were studied.

First of all, the cytotoxic impact of each fusariotoxin was

individually assessed on HepaRG cells using the MTS assay. This method is widely used to study mitochondrial activity changes and consequently cellular metabolism changes, as a result of cytotoxic activity. At the tested concentrations, only DON, ZEA, NIV and T2 decreased cell viability in a dose-dependent manner, with T2 significantly reducing the metabolic activity of HepaRG cells at a 10-fold lower concentration than DON and NIV, and a 100-fold lower dose than ZEA. As a comparison with our previous results obtained from human monocytes THP-1, using the same



Fig. 3. Combination Index (CI)-fraction affected (fa) curves for binary combinations of (A) DON-ZEA and (B) NIV-T2 on HepaRG cells after 48 h exposure, generated by computer simulation from fa = 0.1 to 0.95. CI values were calculated from data obtained from three independent experiments \pm 95% confidence intervals based on SDA using the CompuSyn software. Horizontal dashed lines correspond to lower and upper limits of the additivity zone.



Fig. 4. Effects of 7.35 µM DON, 10 µM MON, 10 µM FB1, 55.1 µM ZEA, 2.84 µM NIV and 0.19 µM T2 alone and in combination on HepaRG cell viability, early apoptosis and necrosis after 3, 6, 12 and 18 h of exposure (mean percentage ± SEM of necrotic cells, early apoptotic cells and viable cells quantified using annexin V-FITC/PI double staining bioassay, N = 3) * and # = cell viability measured mean significantly different from control by significantly inducing * cell apoptosis or # cell necrosis (P < 0.05; ANOVA analysis followed by HSD Turkey's post hoc test).

Effects of 0.2 μ M DON and 20 μ M ZEA alone and in combination on the expression of 7 genes of interest in HepaRG cells after 1 h of exposure (mean \pm SEM fold of control of gene expression quantified using qPCR, N = 3). The expression was arbitrarily set to 1 in control for all measured genes; * and ** = gene expression measured mean significantly different from negative control (*P < 0.05 and **P < 0.01).

				Up-regulation				
Down-regulation								
P<0.01 **	P<0.05 *	P>0.05	P<0.05 *	P<0.01 **				
Gene Name	Control	DON	ZEA	DON-ZEA				
CYP4F3B	1 ± 0.12	1 ± 0.14	0.92 ± 0.57	1.54 ± 0.08				
CYP3A4	1 ± 0.09	0.91 ± 0.23	0.82 ± 0.08	2.27 ± 0.56				
СЕВРа	1 ± 0.06	0.69 ± 0.03	0.89 ± 0.03	0.91 ± 0.03				
HNF4a	1 ± 0.02	0.96 ± 0.04	1.16 ± 0.15	0.94 ± 0.03				
Albumin	1 ± 0.02	1.24 ± 0.02	0.98 ± 0.00	1.48 ± 0.03				
Transferrin	1 ± 0.04	0.82 ± 0.01	0.72 ± 0.05	1.21 ± 0.02				
Aldolase B	1 ± 0.12	0.87 ± 0.13	0.74 ± 0.35	1.35 ± 0.10				



Fig. 5. Effects of 0.2 μ M DON and 20 μ M ZEA alone and in combination after 1 h of exposure on expression of albumin, CYP3A4 and claudin-1 expression. (A) Presented immunoblots are representative of 5 experimental replicates, (B) Mean percentage \pm SEM of protein expression, N = 5, *protein expression measured mean significantly different from negative control (arbitrarily set to 1) (P < 0.05).

mycotoxins as well as identical assay conditions (Smith et al., 2017), the obtained IC₅₀ values were between 1.5 and 30-fold lower for THP-1 depending on the toxins, highlighting a higher sensitivity of monocytes in comparison to hepatocytes. This suggests that different cells representing different organs are differently susceptible to these fusariotoxins. Nevertheless, based on IC₅₀ values, these mycotoxins seemed to affect both THP-1 and HepaRG cells in the same following order: T2 > NIV > DON > ZEA. These results are consistent with data from literature indicating, for instance, that T2 was found to be a strong toxicant compared to other fusariotoxins, while NIV was described as one of the most toxic type B TCT and DON one the weakest ones (Yazar and Omurtag, 2008). On the other hand, the results from combined exposures to DON-MON, DON-FB1, DON-ZEA and NIV-T2 were compared to individual toxins cytotoxicity to investigate if the cytotoxic effects of these fusariotoxins would be enhanced by their combinations. The results showed that, using both RA and IJA models, DON-MON as well as DON-FB1 combinations primarily led to an increased cytotoxicity compared to individual mycotoxins. A similar pattern of this synergistic activity was also observed for the DON-ZEA combination with the 3 applied predictive models. Therefore, DON seems to mainly act synergistically with the tested fusariotoxins when triggering hepatotoxicity. Regarding the NIV-T2 mixture, depending on the concentration combination and the predictive approach, synergism, additivity and antagonism were noticed, highlighting the impact of the tested doses as well as the relevance of the selected model when studying mycotoxin interactions. Some authors simultaneously tested different mathematical models to assess mycotoxin combined effects and obtained different conclusions depending on the approach. For example, Tammer et al. (2007) reported that the combined effect of the doses of citrinin, patulin, ochratoxin and glioxin that individually induced a 20% inhibition of INF_Y production led to synergism based on the IJA model and antagonism based on Loewe's model (also known as concentration addition model). All these models present some advantages and limitations that were discussed in the recent review from Foucquier and Guedi (2015). Despite the simplicity and intuitiveness of the RA model, this method is controversial as it assumes that mycotoxins have linear-dose-effect curves, which is generally not the case. The example of the combined effect study of the sum of several doses of the same mycotoxin, which cannot be synergistic or antagonistic, highlights this point. Regarding the IJA model, it is considered as one of the most popular strategies to predict the combined effects of drugs. Nevertheless, it assumes that the drugs have exponential dose-effect curves. On the other hand, the Chou-Talalay strategy is gaining the interest of an increasing number of researchers (Alassane-Kpembi et al., 2016). However, the dataset needs to fulfil some prerequisites and can only be performed on monotonic doseresponse curves. Therefore, since there is still no reference methodology to characterize mycotoxin interactions, the analysis of their combinations will be facilitated by the collective use of different approaches (Foucquier and Guedj, 2015). Furthermore, while mycotoxin interactions are usually characterized by synergism or antagonism (additivity corresponding to an absence of interaction), it has been shown that more complex response patterns, depending on the tested doses and ratios, may occur. As described by Jonker et al. (2005), when the deviation from the used reference model (e.g. IJA) at low dose levels is different from the deviation at high dose levels, the deviation is characterized as dose leveldependent. On the other hand, when the deviation from the reference model depends on the composition of the mixture (namely the proportion of toxicant 1 compared to toxicant 2 in the case of two substances), it is considered as a dose ratio-dependent deviation. This might explained the different type of interactions observed for the NIV-T2 cocktail depending on the tested doses. However, according to the used mathematical conceptual model, hardly any methods have been developed to detect such deviations. In a previous study, mainly antagonism was observed on THP-1 viability reduction using the same mycotoxin mixtures at concentrations enabling a similar toxicity and using the RA conceptual model (Smith et al., 2017). These findings suggest a higher impact of mycotoxin co-exposure on hepatocytes in comparison to monocytes. Furthermore, while DON and ZEA are generally present simultaneously in food and feed, only few authors have studied their toxicological combined effects in in vitro exposure conditions and conclusions varied considerably from one study to another (Bensassi et al., 2014; Ficheux et al., 2012; Kouadio et al., 2007; Wan et al., 2013). For instance, Bensassi et al. (2014) reported antagonistic effects on human colon carcinoma cells viability (HTC116) after 24 h exposure at high cytotoxic doses (100 μ M DON with 40 μ M ZEA), whereas additive cytotoxicity of DON and ZEA (10 μ M of DON and 20 μ M of ZEA) was observed after 72 h exposure on human epithelial colorectal adenocarcinoma cells (Caco-2) (Kouadio et al., 2007). Nevertheless, our results appeared to be similar to those obtained by Wan et al. (2013) who reported synergism on intestinal porcine epithelial cells viability (IPEC-I2) after 48 h exposure with 2 µM DON and 40 µM ZEA. Noteworthy, most of these studies only focused on cell viability and did not explore cellular mechanisms.

Because these fusariotoxins showed cytotoxicity on HepaRG cells, the cell death mechanisms induced by these toxins alone and in combination were investigated using the annexin V-FITC/PI cell double staining bioassay followed by flow cytometry analysis. This method enables to distinguish viable, apoptotic and necrotic cells based on the high affinity of annexin V for the phosphatidylserine (PS), an important phospholipid cell membrane component existing only in the inner cytoplasm-facing leaflet of the cellular membrane, and the ability of propidium iodide (PI) to bind to the DNA. Staining with annexin V and PI after treatments with fusariotoxins at their IC₅₀ concentrations (or solubility limits for MON and FB1) showed that only FB1 and ZEA significantly reduced cell

viability after 18 h of exposure by inducing both apoptosis and necrosis. Regarding the other fusariotoxins, DON, MON, NIV and T2, they did not seem to significantly induce cell mortality at the selected exposure times (3, 6, 12 and 18 h). These results support the interest in exploring apoptosis and necrosis at longer exposure durations or studying other pathways. Concerning mycotoxin mixtures, no significant cell mortality was observed for DON-MON and NIV-T2, just as for DON, MON, NIV and T2 single exposures. In the same way, DON-FB1 and DON-ZEA seemed to have a similar behavior to FB1 and ZEA single exposures on cell mortality at 18 h. Indeed, these mixtures significantly reduced cell viability at 18 h of exposure (28% and 34% respectively) by inducing both apoptosis and necrosis, just as for FB1 and ZEA single exposures. Thus, no significant difference on cell mortality was observed between these mixtures and FB1 and ZEA individual exposures. Nevertheless, it is worth noting that DON-FB1 and DON-ZEA induced a significant cell mortality rate at 3 h (27%) and 12 h (29%) respectively, whereas individual mycotoxin exposures did not show any significant differences with the control at the same exposure durations, highlighting an earlier effect of these two mixtures on cell mortality compared to individual exposures. The induced mortality by either DON-FB1 or DON-ZEA at 6 h was not significant in comparison to the corresponding single exposures. Apoptosis induction observed with the DON-FB1 combination at 3 h of exposure and then after 18 h of exposure could be explained by the possible reversible character of PS externalization (Geske et al., 2001). A comparison with our previous results obtained from human monocytic THP-1 cell lineage showed that similar cell death mechanisms were triggered by these fusariotoxins on human monocytes, such as induction of both apoptosis and necrosis by FB1 and ZEA alone and in combination with DON, as well as no cytotoxic effect of DON, MON and NIV individual exposures at the selected times. Nevertheless, T2 as well as NIV-T2 combination seemed to induce necrosis in THP-1 cells while no significant effect was observed for these treatments in the present study (Smith et al., 2017). Thus, although apoptosis and/or necrosis induction appeared earlier or later for some toxins depending on the considered cell model, our findings showed that there are cell death mechanisms inherent to each toxin. Several authors have been interested in cell death mechanisms induced by mycotoxin single and co-exposures, but many of them conducted their studies during 24 h or more. For instance, using the same annexin V-FITC/propidium iodide cell double staining method, Lei et al. (2013) showed that 24 h incubation with DON (1, 2 and 4 μ M) induced apoptosis on porcine kidney 15 cells in a concentration-dependent manner and Ren et al. (2015) demonstrated that DON (0.674–168.5 μ M) mainly induced apoptosis in a dose dependent-manner after 48 h exposure on chicken splenic lymphocytes. Nevertheless, Goossens et al. (2012) did not observe any significant difference for apoptotic cells on intestinal porcine epithelial cells derived from the jejunum after 72 h exposure, compared to the control condition at the lowest tested DON dose (3.37 µM), close to the tested concentration in our study. However, a comparison with other studies is difficult as PS externalization during apoptosis is mainly cell line-dependent (Lee et al., 2013). Other parameters are commonly used to study cell apoptosis or necrosis after mycotoxin single and combined exposures, such as DNA fragmentation, cell cycle analysis and expression, as well as the activation of key proteins involved in signaling cascades controlling apoptosis (i.e. caspase-3, MAPKs, p-53, Bcl-2, etc.) for apoptosis detection, and the release of lactate dehydrogenase (LDH) activity for necrosis detection. In general, the capacity of DON to induce apoptosis in different cell lines and tissues has already been demonstrated by several authors (Kouadio et al., 2007; Minervini et al., 2004; Pestka et al., 2005, 2004). Furthermore, other major fusariotoxins, such as FB1 and ZEA, have also been reported to

induce apoptosis. For example, Ayed-Boussema et al. (2008) showed that ZEA induced apoptosis in a dose-dependent manner in HepG2 cells via a p53-dependent mitochondrial pathway. Moreover, Kouadio et al. (2007) observed an increase in DNA fragmentation after 24 h of individual and combined exposures of DON (10 μ M), FB1 (10 μ M) and ZEA (10 μ M) on human Caco-2 cells, with mycotoxin combinations leading to higher DNA fragmentation, and suggested the occurrence of apoptosis. In addition, Bensassi et al. (2014) demonstrated that cell mortality after 48 h of DON and ZEA single and combined exposures induced apoptosis on HTC116 human colon cells by involving the mitochondrial intrinsic pathway.

Because of the high occurrence of the DON-ZEA mixture in cereals all over the world (Smith et al., 2016), the second part of this study focused on these 2 mycotoxins to compare the effects of single and combined exposures from a mechanistic cellular response point of view. In particular, to obtain a better view of the potential early impact of mycotoxin mixtures on HepaRG functional state and metabolic pathways, the expression of relevant hepatocyte-specific factor associated genes was measured in HepaRG cells exposed to DON and/or ZEA during 1 h at their respective IC₁₀. This inhibitory concentration was selected for both mycotoxins assuming that such a dose would potentially induce a significant cellular response that can be observed at the transcriptome level while limiting cell mortality. Contrary to other human liver cell lines, HepaRG cells maintain many liver-specific functions with expression levels close to those found in primary human hepatocytes, including the expression of major cytochromes P450s, membrane transporters, nuclear receptors and Phase II enzymes (Andersson et al., 2012; Aninat et al., 2006; Anthérieu et al., 2012, 2010; Gerets et al., 2012; Jossé et al., 2008). The selected genes corresponded to the following liver-specific factors: the humanrelevant cytochrome P450 CYP3A4 and CYP4F3B enzymes involved in phase I detoxification metabolism, the nuclear C/EBPa and HNF4a transcription factors, some liver-specific proteins such as transferrin and albumin plasma proteins and the aldolase B glycolytic enzyme. Indeed, liver is the main source of major P450 enzymes, CYP3A4 being the most important and abundant one expressed in the human liver, as it contributes to the metabolism of approximately 50% of the drugs in use today (Guengerich, 1999). On the other hand, the CYP4F3B isoform, which is a human liverspecific cytochrome P450 belonging to the CYP4F family and the predominant isoform in the liver, may exert important functions in lipid homeostasis and in inflammatory diseases (Antoun et al., 2008). Most of P450 expression are under strict control of various transcription factors that are regulated during differentiation (Burk and Wojnowski, 2004). For instance, the hepatocyte nuclear factor 4α (HNF4 α) and the liver-enriched nuclear factor CCAAT/enhancerbinding protein (C/EBP) α are key regulators controlling the expression of several hepatic metabolic processes and thus are potentially entailed in the regulation of these P450 enzymes. More particularly, HNF4 α is a critical regulator for a large number of hepatic specific genes involved in lipid and carbohydrate metabolism, while C/EBP α is a master regulator for key glyconeogenic enzymes and is essential for energy homeostasis (Darlington et al., 1995; Petrescu et al., 2002). In addition, Ourlin et al. (1997) reported that C/EBP α plays a prominent part in the expression of the CYP3A family and Burk and Wojnowski (2004) highlighted that HNF4a was considered to be one the most important regulator of the xenobiotic-induced regulation of CYP3A genes, including CYP3A4 which constitutes a direct target of HNF4a. Furthermore, Madec et al. (2011) reported that the differentiation process of HepaRG cells was characterized by a gradual increased expression of HNF4a, CYP3A4, CYP4A11 and CYP4F3B as well as specific enzymes of glucose metabolism such as aldolase B, highlighting that

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various P450 might share common mechanisms controlling gene expression mediated by liver-specific transcription factors and nuclear receptors. Furthermore, a reduction of the expression of the CYP genes or of the other above-mentioned genes might highlight a loss of specialized liver functions or, in the case of differentiated cells, could even suggest a dedifferentiation of hepatocytes (Godoy et al., 2009). On the contrary, an increase of the expression of these genes could indicate an 'overactivity' of the cells. Herein, our results showed that the cells co-exposed to DON and ZEA induced more expression changes compared to their single exposures. Indeed, the mycotoxin mixture significantly increased the expression of most of the studied genes, including those encoding for the drugmetabolizing enzymes CYP3A4 and CYP4F3B, highlighting a high metabolic activity of the cells after only 1 h of exposure at low cytotoxic doses. Thus, given the essential role of CYP3A4 in the metabolism of the drugs, and because the liver is the main detoxifying organ of our organism (Guengerich, 1999), HepaRG cells exposed to the mycotoxin mixture, and which appeared 'overfunctional', could metabolize some drugs more quickly. Therefore, this 'overactivity' of hepatocytes can be problematic for human and animal health since some toxic drug metabolites could accumulate in the cells when DON and ZEA are present together. These findings might suggest possible interferences between mycotoxins and drugs for patients following a drug therapy, which would be especially problematic when the pharmacological activity of the drug lies within the parent molecule. Moreover, despite the increase of the expression levels of genes encoding for CYP3A4 and CYP4F3B after DON-ZEA combined exposure, the expression of the two transcription factors C/EBP α and HNF4 α did not seem to be affected by the mixture. Yet, CYP3A4 is known to be under control of these two liver nuclear factors (Burk and Wojnowski, 2004; Ourlin et al., 1997). In differentiated HepaRG cells, Madec et al. (2011) observed a correlation between HNF4a activation and the regulation of CYP3A4 expression but not with CYP4F3B expression. These findings support the interest in exploring the expression of other nuclear receptors such as the retinoid X receptor, the constitutive androstane receptor (CAR) or the pregnane X receptor (PXR) which is considered, with HNF4a, to be one the most important modulators of xenobiotic-induced regulation of the CYP3A gene (Burk and Wojnowski, 2004).

In order to study the possible regulation of some of the above mentioned liver-specific factors at a translational level, the expression of albumin and CYP3A4 was also measured by Western blot. Interestingly, despite higher mRNA levels for albumin in the cells exposed to DON alone and in combination with ZEA, low protein levels were measured by Western blot after DON individual exposure only. This might suggest that albumin has been regulated at the post-transcriptional level. In general, albumin synthesis of hepatocytes is a marker for liver-specific functions. Thus, the decrease in albumin expression observed herein is a sign for the disturbance of cellular functions by DON. Königs et al. (2008) reported the decrease in albumin secretion into the medium by human hepatocytes (primary hepatocytes and HepG2 cell line) after DON exposure. On the other hand, as no significant effect was observed on the albumin expression when DON was present simultaneously with ZEA, we hypothesized that ZEA could interfere with DON effects, preventing the potential post-translational regulation of albumin induced by the latter. In this context, it would be interesting to investigate the signaling pathway modulation by these two mycotoxins, individually and in combination, using untargeted "omic" approaches that allow for a finer mechanistic comprehension. Regarding CYP3A4, the results were comparable to the gene expression level measurement, namely a significant increase of the expression of this drug-processing enzyme after DON-ZEA combined exposure only. Alteration of

liver functions being one of the most commonly described effects of acute mycotoxin exposure, which in extreme cases may lead to death (Pitt, 2000), several authors have been interested in mycotoxin-induced hepatotoxicity. For instance, the hepatotoxicity of aflatoxin B1, which is an extensively studied mycotoxin, has been reported to be CYP3A4-dependent (Gallagher et al., 1996; Sivertsson et al., 2010; Ueng et al., 1995). More specifically, the toxicity of aflatoxin B1 is mediated by the formation of the toxic metabolite 8,9-epoxide, formed by CYP1A2 and 3A4, which justifies the higher sensitivity of differentiated HepaRG cells to this toxin, compared to undifferentiated cells which poorly express major cytochromes P450s (Guillouzo et al., 2007). Here, the elevated mRNA expression levels associated with an increase in CYP3A4 protein expression suggested a CYP3A4-dependent catalytic activity induced by a DON-ZEA treatment. In addition, mRNA expression levels also suggested the involvement of CYP4F3B in the DON-ZEA combination-induced toxicity. Finally, regarding the tight-junction protein claudin-1, its expression appeared to be reduced after the DON-ZEA co-exposure compared to the control suggesting that the mixture could impair the cell tissue permeability after only 1 h of exposure. Thus, these findings indicated that cells exposed to mycotoxins, especially in combinations, could be affected at a molecular level even at low cytotoxic concentrations (as measured by viability assessments) and after a short exposure time. However, it is worth noting that both mRNA and protein analyses were performed at the same time (1 h); therefore, it would be of interest to perform kinetic studies of mRNA and corresponding proteins to have a better view of their related transcription and translation regulations in this context.

5. Conclusion

In this study, we reported the synergism of all tested fusariotoxin mixtures, mainly at low cytotoxic doses, on hepatocyte viability reduction suggesting that the simultaneous presence of fusariotoxins in food commodities may be more toxic than the presence of mycotoxins alone. In addition, we investigated the cellular mechanisms involved in the response to mycotoxin single and combined exposures, where synergism was also observed at the gene level for the DON-ZEA combination. Nevertheless, in order to observe significant cellular responses to mycotoxin exposures in acute exposure conditions, we used mycotoxin concentrations above the maximum limits permitted by the European regulations in food and feedstuffs. Thus, besides these results, further investigations regarding the study of mycotoxin toxicological effects at subtoxic concentrations and in chronic exposure conditions (closer to real food consumption habits) should be carried out to provide additional data on health risk assessment for the current co-exposure to mycotoxins to the scientific community and regulatory agencies.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Les points marquants de cette étude sont les suivants :

- Les mycotoxines DON, ZEA, NIV et T2 réduisent la viabilité des cellules HepaRG d'une manière dose-dépendante, T2 étant la plus cytotoxique. En revanche, aucune cytotoxicité n'est observée pour MON et FB1 aux doses testées après 48h d'exposition.
- Toutes les combinaisons de mycotoxines testées montrent des effets combinés synergiques sur la viabilité cellulaire après 48h d'exposition, quel que soit le modèle mathématique utilisé.
- Seules la FB1 et la ZEA, individuellement et en mélange avec le DON, semblent induire l'apoptose et la nécrose des cellules, essentiellement après 18h d'exposition.
- A l'échelle moléculaire, après seulement 1h d'exposition, le mélange DON+ZEA induit l'expression de certains gènes codant pour des enzymes impliquées dans la dégradation des xénobiotiques, contrairement au DON et à la ZEA en mono-exposition.

La figure 8 schématise de façon synthétique les principaux résultats obtenus dans cette étude.



Figure 8 : Représentation schématique des principaux effets biologiques observés dans l'étude 2.



Figure 8 (suite) : Représentation schématique des principaux effets biologiques observés dans l'étude 2.

2. Etude 2 : Etude des modifications du protéome global des cellules HepaRG induites par l'exposition individuelle et combinée au déoxynivalénol et à la zéaralènone

Etude n°3:

Differential impacts of individual and combined exposures of deoxynivalenol and zearalenone on the HepaRG human hepatic cell proteome

Journal of Proteomics (https://doi.org/10.1016/j.jprot.2017.11.025)

Marie-Caroline Smith, Emma Timmins-Schiffman, Nolwenn Hymery, Monika Coton, Emmanuel Coton, Brook Nunn, Stéphanie Madec

Au vu des premiers résultats obtenus d'un point de vue moléculaire sur la réponse cellulaire des HepaRG à l'exposition au DON et/ou à la ZEA, nous avons souhaité utiliser une approche non-ciblée permettant de mettre en évidence de façon plus fine les mécanismes de cette réponse cellulaire. Dans ce contexte, la deuxième étude de cette seconde partie s'intéresse tout particulièrement aux effets du DON et de la ZEA, seuls et en mélange, sur le protéome global des HepaRG dans un état indifférencié. A partir des profils protéomiques obtenus, il est possible, grâce aux nombreux outils d'analyse protéomique disponibles aujourd'hui, d'identifier les principaux processus biologiques impliqués dans la réponse cellulaire à l'exposition et la co-exposition à ces mycotoxines.

Ainsi, nous avons choisi d'exposer les cellules HepaRG (non différenciées) au DON, à la ZEA et au mélange DON+ZEA pendant 1h et 24h aux CI_{10} obtenues dans l'étude précédente après une exposition de 48h avec ces mycotoxines. En effet, il a pu être observé dans l'étude précédente que l'expression de quelques protéines ciblées (telles que le CYP3A4 et l'albumine), spécialisées dans la fonction des hépatocytes, semblait être modifiée après seulement 1h d'exposition à ces faibles concentrations cytotoxiques, avec un effet plus important du mélange que des toxines seules. De plus, dans la première partie sur les THP-1, nous avons également pu observer des changements d'expression pour certaines protéines des

voies de signalisation impliquées dans la réponse au stress des cellules (MAPKs) après 1h d'exposition aux CI₁₀. C'est pourquoi nous sommes partis de l'hypothèse qu'il était probable que d'autres protéines étaient impliquées dans les modifications fonctionnelles liées à l'effet toxicologique des mycotoxines, même après seulement 1h d'exposition. Par ailleurs, la CI₁₀ obtenue à 48h *via* un test MTS nous semblait particulièrement adaptée à ce type d'analyse. En effet, une telle dose pourrait induire une réponse cellulaire significative, facilement observable au niveau du protéome, tout en limitant la mortalité cellulaire afin d'éviter des événements strictement liés à la mort des cellules qui pourraient nuire à l'analyse protéomique.

L'objectif de cette étude était de pouvoir comparer i) les effets individuels et les effets combinés du DON et de la ZEA à un temps d'exposition donné ainsi que ii) les effets précoces (après 1h d'exposition) et les effets plus tardifs (après 24h d'exposition) induits par ces fusariotoxines sur le protéome des cellules. Pour ce faire, le protéome des cellules HepaRG exposées au DON, à la ZEA et au mélange DON+ZEA a été analysé par LC-MS/MS et comparé à celui de cellules non exposées aux mycotoxines. La LC-MS/MS est un outil d'analyse couramment utilisé aujourd'hui en protéomique car il permet de détecter les protéines d'un mélange, même présentes en très petites quantités, de les identifier et de les quantifier. En particulier, l'outil d'analyse utilisé dans cette étude, une LC-MS/MS Q-Exactive, fait partie des outils les plus résolutifs.

Par ailleurs, dans le but de connaître la proportion absorbée et/ou dégradée de ces mycotoxines par les cellules, nous avons mesuré les concentrations en DON et ZEA présentes dans le milieu de culture des HepaRG à la fin des périodes d'exposition avec ces mycotoxines (après 1h et 24h). Les surnageants de culture ont été analysés par Q-TOF (*Quadrupole Time-of-Flight*) LC-MS et les concentrations en DON et ZEA ont pu être déterminées à partir de gammes étalons des molécules standards.

La figure 9 schématise de façon synthétique les principaux effets biologiques évalués dans cette étude.

Les données supplémentaires associées à cet article sont présentées en annexe II.



Figure 9 : Résumé schématique des différents effets biologiques et conditions évalués dans l'étude n°3.

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Differential impacts of individual and combined exposures of deoxynivalenol and zearalenone on the HepaRG human hepatic cell proteome

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ABSTRACT

Numerous surveys have highlighted the natural co-occurrence of deoxynivalenol (DON) and zearalenone (ZEA) mycotoxins in food and feed. Nevertheless, data regarding cellular mechanisms involved in response to their individual and simultaneous exposures are lacking. In this study, in order to analyze how low mycotoxin doses could impact cellular physiology and homeostasis, proteomic profiles of proliferating human hepatic cells (HepaRG) exposed for 1 h and 24 h to low DON and ZEA cytotoxicity levels (0.2 and 20 µM respectively), alone or in combination, were analyzed by LC-MS/MS. Proteome analyses of mycotoxin-treated cells identified 4000 proteins with about 1.4% and 3.7% of these proteins exhibiting a significantly modified abundance compared to controls after 1 h or 24 h, respectively. Analysis of the Gene Ontology biological process annotations showed that cell cycle, proliferation and/or development as well as on DNA metabolic processes were affected for most treatments. Overall, different proteins, and thus biological processes, were impacted depending on the considered mycotoxin and exposure duration. Finally, despite the important proteome changes observed following 24 h exposure to both mycotoxins, only the uptake of ZEA by the cells was suggested by the mycotoxin quantification in cell supernatants.

Biological significance: This study investigated the proteomic changes that occurred after DON and ZEA (individually and in combination) short exposures at low cytotoxicity levels in proliferating HepaRG cells using LC-MS/MS. The obtained results showed that the cellular response is time- and mycotoxin or mixture-dependent. In particular, after 1 h exposure, the DON + ZEA combination led to more proteomic changes than DON or ZEA alone, whereas the opposite was observed after 24 h. In addition, the significant cellular response to stress induced by ZEA after 24 h exposure seemed to be reduced when combined with DON. Thus, these results supported a possible mitigation by the hepatocytes when exposed to the mycotoxin mixture for a long duration. These findings represent an essential step to further explore adaptive cell response to mycotoxin exposure using with more complex incubation kinetics and combining different "omics" tools. Moreover, as mycotoxin quantification in cell supernatants showed different behaviors for DON and ZEA, this also raises the question about how mycotoxins actually trigger the cell response.

1. Introduction

The deoxynivalenol (DON) and zearalenone (ZEA) mycotoxins are both secondary metabolites produced by some Fusarium species, such as F. culmorum, F. crookwellense and F. graminearum, that can infect cereal crops under certain environmental conditions [1]. These mycotoxins are among the most widely distributed fusariotoxins in grains, respectively present in 59% and 45% of the cereal samples analyzed worldwide between 2009 and 2011 [2]. In addition, they are commonly found to co-occur in food commodities, mainly in the North Temperate Zone of the world [3].

DON, a type B trichothecene (TCT), is a polar organic compound

Abbreviations: α-ZAL, α-zearalanol; α-ZOL, α-zearalanol; β-ZAL, β-zearalanol; β-ZOL, β-zearalanol; DOM-1, de-epoxy deoxynivalenol; DON, deoxynivalenol; ESI, electrospray ionization; ER, endoplasmic reticulum; IC, inhibitory concentration; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MAPKs, mitogen-activated protein kinases; MS, mass spectrometer; MTS, mitrochondrial tetrazolium salt; QC, quality control; Q-TOF, quadrupole time-of-flight; TCT, trichothecenes; ZEA, zearalenone

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containing 3 free hydroxy groups (- OH) associated with its toxicity [4]. In several animal species, consumption of high doses of DON mainly causes acute temporary nausea, emesis, diarrhea, abdominal pain, headache, dizziness and fever, while chronic exposure to small doses elicits anorexia, growth retardation, impaired reproduction (reduced litter size) and adverse effects on the thymus, spleen, heart and liver [4–6]. A primary target of this mycotoxin is the innate immune system [7]. ZEA is classified as a non-steroidal estrogen. This fusariotoxin activates the estrogen gene and causes functional and morphological alterations on reproductive organs [8,9]. In particular, its exposure can lead to various estrogenic effects, such as decreased fertility, increased embryolethal resorption, reduced litter size, changed weight of adrenal, thyroid and pituitary glands and change in serum levels of progesterone and estradiol [10]. Furthermore, ZEA is mainly metabolized in the liver, which also seems to be a main target for the toxin. While DON and ZEA are generally simultaneously present in food and feed, only few authors have studied their combined toxicological impacts in in vitro conditions. To date, data about the cellular mechanisms involved in the response to individual and combined exposures of DON and ZEA are still limited. This is likely because most of the studies use targeted analytical approaches and very few employ high-throughput, discovery-based methods (such as proteomics). The liver being our main detoxification organ, human hepatocytes represent one of the most relevant in vitro models for toxicity and cellular response studies.

In this study, we investigated the proteomic changes associated with DON and ZEA (individually and in combination) short exposures at low cytotoxicity levels in proliferating HepaRG cells, a cell line derived from a human hepatocellular carcinoma, using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2. Materials and methods

2.1. Mycotoxins

Mycotoxin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): DON (CAS#51481-10-8; purity > 98%) and ZEA (CAS#17924-92-4; purity > 99%). Standards were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) to final concentrations of 8×10^{-5} M for DON and 8×10^{-3} M for ZEA, and stored at -20 °C.

2.2. Cell and culture conditions

Human hepatocytes (HepaRG) were acquired from Biopredic International (Saint Grégoire, France). They were cultured in William's E medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin and 50 µM hydrocortisone hemisuccinate at 37 °C with 100% relative humidity (RH) in a 5% CO₂ atmosphere. Culture medium was renewed every 2 to 3 days with fresh growth medium. The cells were passaged every 2 weeks at a density of 2.7×10^4 cells/cm² by a short time exposure (< 5 min) with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Sigma-Aldrich), and reseeded in a fresh medium.

For proteomic interrogations, biotriplicate samples of human hepatic cells were prepared by exposing 3×10^6 proliferating HepaRG cells, previously maintained two days at confluence in 25 cm² flasks, for 1 h and 24 h to DON (0.2 μ M) and ZEA (20 μ M) alone and in combination. The selected doses corresponded to 10% growth inhibition concentrations (IC₁₀) previously determined using a MTS cytotoxicity assay after 48 h exposure [11]. The final concentration of DMSO in cell cultures containing mycotoxins was maximum 0.5%. Appropriate control cultures without mycotoxin, but containing the same amount of solvent, were included as controls.

2.3. Proteomic sample preparation for tandem mass spectrometry analysis (LC-MS/MS)

Cellular preparations for proteomic analyses were completed as follows: cells were harvested and washed twice in ice-cold PBS by centrifugation at 400 \times g for 10 min at 4 °C. Supernatants were removed and pellets were lysed in 100 µl of 50 mM ammonium bicarbonate with 8 M urea on ice. Protein concentrations were measured on each sample using the Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). Samples were diluted to yield a final concentration of 1 μ g protein μ l⁻¹ and 100 μ l of the obtained solutions were transferred to new tubes for digestion. Protein disulfide bonds were reduced with 6.6 ul of 1.5 M tris pH 8.8 and 2.5 ul of 200 mM tris (2-carboxyethyl) phosphine (37 °C, 1 h). For protein alkylation, 20 µl of 200 mM iodoacetamide was added and samples were then vortexed, and incubated for 1 h at room temperature in the dark. To absorb excess iodoacetamide, 20 µl of 200 mM dithiolthreitol was added, followed by vortexing and incubating at room temperature for 1 h. Samples were diluted with 800 µl of 25 mM ammonium bicarbonate and 200 µl of methanol, followed by digestion with Promega Trypsin (1:20; enzyme:protein) overnight at 37 °C. Samples were then evaporated on a SpeedVac to near dryness. Dilute formic acid (100 µl, 0.1%) was added to reduce the pH to < 2 and the samples were evaporated to dryness using a SpeedVac. Prior to mass spectrometry, samples were desalted using Microspin C18 columns following manufacturer's guidelines (Nest Group, Southborough, MA, USA). After desalting, the remaining solvent was evaporated with a SpeedVac. Finally, peptide samples were resuspended in 5% acetonitrile in 0.1% formic acid and frozen at - 80 °C until LC-MS/MS analyses were performed.

2.4. Mass spectrometry analyses

Samples were separated and introduced into the mass spectrometer (MS) by reverse-phase chromatography using a 30 cm long, 75 µm i.d., fused silica capillary column packed with C18 beads: Reprosil-Pur C18-AQ 3 µm (Dr. Maisch GmBH, Ammerbuch, Germany), and fitted with a 2 cm long, 100 µm i.d. precolumn (C18-AQ 3 µm Dr. Maisch GmBH). Peptides were eluted using an acidified (formic acid, 0.1% v/v) wateracetonitrile gradient (5-35% acetonitrile in 60 min). Mass spectrometry was performed on a Thermo Fisher (San Jose, CA) Q-Exactive (QE). The top 20 most intense ions were selected for MS2 acquisition from precursor ion scans of 400–1400 mz⁻¹. Quality control (QC) peptide mixtures (Pierce mixed peptide PRTC standards) were analyzed every 12th injection to monitor chromatography and MS sensitivity. Skyline was used to determine that QC standard retention time and isotopic distribution did not deviate > 10% through all analyses [12]. For quantitative analyses, biotriplicate samples from cells exposed to either DMSO, DON, ZEA or DON + ZEA were analyzed on the QE in technical triplicates using data-dependent acquisition (DDA), culminating in a total of nine analyses per treatment. Raw data have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications (PRIDE) [13] partner repository with the dataset identifiers PXD005840 (for 1 h exposure experiments) and PXD006267 (for 24 h exposure experiments).

2.5. Protein database searching and MS data interpretation

Following methods detailed by Nunn et al. [14], all tandem MS results were searched and interpreted with COMET v. 2016.01 rev. 2 [15,16], an open source tandem mass spectrometry (MS/MS) sequence database search engine for peptide identification. The protein database used for correlating spectra with protein identifications was a recent Human database consisting of 21,030 proteins (Human fasta 10-22-2015 from Swiss-prot database with isoforms at uniprot.org). Then, data were analyzed using the Trans-Proteomic Pipeline, which includes PeptideProphet and ProteinProphet [17], and then tabulated by

ABACUS, a computational tool for extracting and pre-processing spectral counts from MS/MS data sets, which aggregates data across replicates [18]. Only proteins with > 1 peptide and > 90% probability were retained for final data interpretations. The common method of spectral counting was selected to determine relative protein abundance. Spectral counting sums up the number of identified peptide tandem mass spectra resulting from a specific protein in order to estimate abundance of that protein relative to other proteins in the sample. Proteomics data were interrogated at the protein level using QSpec to determine relative quantities of proteins observed between control conditions and the different treatments (i.e. DON, ZEA and DON + ZEA) [19] (http://www.nesvilab.org/qspec.php/). QSpec was designed specifically for analyzing differential protein abundance data using label-free tandem mass spectrometry spectral counts. QSpec is reported using a fold change difference in abundance on a log base 2 scale. A reported positive fold change indicates a significant increase in abundance and a negative fold change indicates a significant decrease in abundance, while a reported fold change of zero indicates no significant difference between the treatments. Proteins were considered to be significantly increased or decreased in abundance if the reported Z score was $\geq |2|$ and the fold difference observed was $\geq |0.5|$. Then, the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) was used to identify significant biologically enriched processes within the large data set [20,21]. Proteins that were determined to be at significantly greater or lower abundance in response to each treatment condition were examined using this functional annotation tool. All 4000 proteins identified across biological and technical replicates were used as the background protein list.

2.6. DON and ZEA quantification in cell supernatants by LC-MS/QTOF

Supernatants from HepaRG cell cultures exposed 1 and 24 h to DON, ZEA and DON + ZEA mixture, as well as control cells, were collected and stored at -20 °C until metabolite quantification. Metabolites were directly extracted from supernatants by dissolving in LC-MS grade acetonitrile (1:1), prior to filtration through a 0.2 µm PTFE membrane syringe 4 mm filter (GE Healthcare Life Sciences, Little Chalfont, UK) into an amber vial. A LC-MS/Quadrupole Time-of-Flight (Q-TOF) was used in order to separate and identify the extracted metabolites from each sample. The Agilent 1290 Series HPLC system included a binary pump and degasser, well plate autosampler with thermostat and a thermostat-capable column compartment. Two microliters of each sample were injected in the system and separation was achieved using a ZORBAX Extend-C18 column (2.1 \times 50 mm and 1.8 µm, maximum pressure 600 bar) (Agilent, France) that was maintained at 35 °C throughout the chromatographic run. The flow rate was set to 0.3 ml min⁻¹ using the following mobile phase: solvent A (milli-O water + 0.1% formic acid + 10 mM ammonium formate) and solvent B (100% acetonitrile). Solvent B was maintained at 5% for the first 4 min, followed by a gradient of 5-100% of solvent B for 16 min, and then maintained at 100% during a 5-min post-time to equilibrate the column to original run conditions. Metabolites were detected using an Agilent 6530 Series Accurate-Mass Q-TOF mass spectrometer with an electrospray ionization (ESI) source operated in positive and negative ion modes. Mass spectrometer conditions were as follows: capillary voltage, 4 kV; source temperature, 325 °C; nebulizer pressure, 50 psig; drying gas, 121 min⁻¹ and ion range, 100–1000 m/z. LC-MS/QTOF calibrations were performed before each run following the mass spectrometer manufacturer's instructions. Relative DON and ZEA quantifications were carried out using the prepared mycotoxin standards previously described. For quantification, an 8 point linear range from 0.01 to 50 µM for both mycotoxins was prepared in acetonitrile. Some points were also prepared in the culture medium diluted in acetonitrile (1:2) and no matrix interference was observed. DON could be detected using the $[M-H]^+$ 297.133 m/z ion and $[M-Na]^+$ 319.115 m/z ion in ESI + mode while ZEA quantification was performed using the [M-H⁺]⁻

Table 1

LC-MS/QTOF method performance characteristics for mycotoxin quantification in supernatants.

Compound - formula	Retention time (min)	Quantifier ion (<i>m</i> / <i>z</i>)	Qualifier ion (m/z)	\mathbb{R}^2	ESI mode
DON - C15H20O6	1.33	319.1151	297.1328	0.9925	+
ZEA – C ₁₈ H ₂₂ O ₅	13.20	317.1392	n/a	0.9986	-

317.139 m/z ion in ESI- mode. All metabolite characteristics used for LC-MS/QTOF analyses can be found in Table 1.

3. Results

3.1. HepaRG proteomic profiles following DON and/or ZEA exposures

To determine the mechanisms supporting the observed cytotoxicity of DON and ZEA in HepaRG cells [11], the proteomic profile changes induced by the individual and combined exposures to these two fusariotoxins after 1 h and 24 h were investigated by LC-MS/MS analysis. Proteomes of control cells as well as DON-, ZEA- and DON + ZEAtreated cells yielded the identification of 4000 inferred proteins with two or more unique peptides, representing about 19% of the human proteome (based on the human proteome from Swiss-prot database with isoforms (22-10-2017) consisting of 21,030 proteins).

After only 1 h treatment with DON, ZEA and DON + ZEA, the mixture DON + ZEA seemed to impact more proteins than single mycotoxins. Indeed, 21, 6 and 35 proteins exhibited a significantly enhanced or reduced abundance compared to control experiments, respectively (Z score $\geq |2|$ and observed fold difference $\geq |0.5|$) (Supplementary data, Table S1). Regarding the 21 proteins affected by DON, 7 were at higher abundances and 14 at lower abundances than in the control condition, while among the 6 proteins affected by ZEA, 5 were detected at higher levels and only 1 at a lower level than in the control. For the 35 DON + ZEA-response proteins, 6 exhibited a higher abundance and 29 a lower abundance compared to the control. The comparison of the proteomes of cells exposed 1 h to DON, ZEA and DON + ZEA showed that none of the identified proteins were common to DON- and ZEA-treated cells (and thus to DON-, ZEA- and DON + ZEA-treated cells), and very few were shared between cells co-exposed to DON + ZEA and cells exposed to DON and ZEA individually (Table 2 and Fig. 1A). More specifically, five DON-response proteins were also affected by the combination: the DNA topoisomerase 1 (P11387), DNA topoisomerase 2-alpha (P11388), DNA topoisomerase 2-beta (Q02880), RNA-binding protein 28 (Q9NW13) and tyrosineprotein kinase BAZ1B (Q9UIG0) and, for ZEA-response proteins, 2 proteins were also differentially abundant in DON + ZEA: the glyoxalase domain-containing protein 4 (Q9HC38) and carbamoyl-phosphate synthase [ammonia] (mitochondrial) (P31327), representing about 24% and 33% of the proteins that responded to DON and ZEA respectively (Table 2). In addition, the five identified proteins common to DON and DON + ZEA after 1 h exposure were at lower abundances than in the control whereas the two proteins common to ZEA and DON + ZEA were at higher abundances. Thus, a total of 55 proteins with a significant difference in abundances were identified from HepaRG cells after 1 h exposure with treatments. Among these 55 proteins, 39 were at a lower abundance than in the control treatment (representing 75%).

Regarding the 24 h exposure, ZEA treatment seemed to impact many more proteins than the DON or mixture conditions. After 24 h exposure to DON, ZEA and DON + ZEA 55, 96 and 39 proteins with a significantly modified abundance compared to the control treatment were identified, respectively (Supplementary data, Table S1). Concerning the 55 DON-response proteins, 5 were at higher abundances and 50 were at lower abundances while among the 96 proteins affected

Common proteins to the different treatments (\mathcal{A} = higher abundance proteins; λ = lower abundance proteins)

Table 2

	ZEA 24 h 12 proteins ∖a: P53522, Q13576, Q15418, Q6NT55, Q8N5N7, Q8NE71, P06756, P11387, P11388, P32321, P62834, Q86UP2
	DON 24 h 19 proteins \: A6NIZ1, 060271, P00505, P02795, P08183, P11233, P11234, P14678, P20073, P27487, P45973, Q8WWI1, Q969X5, P06756, P11387, P11388, P32321, P62834, Q8GUP2 18 proteins: 2 /: P31350, Q9598 16 \: P01024, P02751, P0C014, P11717, P16401, P42166, P50402, Q07954, Q96989, Q9BUJ2, P06756, P11387, P11388, P32321, P62834, Q8GUP2
	DON + ZEA 1 h 2 proteins ∖: 7 p11387, p11388 5 proteins \: 911387, p11388, Q13724, Q8NE71, 921695 (P21695 / for DON + ZEA) 3 proteins \: Q8NE71, P11387, P11388
	ZEA 1 h 2 proteins /: Q9HC38, P31327 1 protein \: P45973 0 protein
DON 1 h	0 protein 5 proteins \: P11387, P11388, Q02880, Q9NW13, Q9UIG0 2 proteins \: P11387, P11388 3 proteins \: P11387, P11388, Q6P179 2 proteins \: P11387, P11388
	ZEA 1 h DON + ZEA 1 h DON 24 h ZEA 24 h DON + ZEA 24 h

by ZEA, 9 were detected at higher levels and 87 at lower levels compared to the control treatment. Similarly, for the mixture, most of the 39 response proteins exhibited lower abundances than in the control condition (4 with an enhanced abundance and 35 with a reduced abundance). Furthermore, 18 DON-response proteins and 12 ZEA-response proteins were also affected by DON + ZEA, which represented 33% and 13% of the DON- and ZEA-affected proteins, respectively (Table 2 and Fig. 1B). After this exposure duration, 6 proteins were common to all treatments, namely the deoxycytidylate deaminase (P32321), DNA topoisomerase 1 (P11387), DNA topoisomerase 2-alpha (P11388), integrin alpha-V (P06756), kinectin (O86UP2) and Ras-related protein Rap-1A (P62834). These 6 proteins were all at a lower abundance than in the control. They represented about 4% of the 147 identified proteins from HepaRG cells displaying a significant difference in abundances (Table 2). Among these 147 proteins, 131 were at lower abundance (representing 89%), and all of those common to DON, ZEA and DON + ZEA were mostly detected at lower levels than in the control (Fig. 1B).

A comparison between 1 h and 24 h exposure showed that the number of proteins with a significantly altered abundance compared to the control highly increased in HepaRG cells after 24 h exposure with single mycotoxins, particularly with ZEA, while the number of impacted proteins remained stable with the mixture. Between the two treatment periods, we only observed two proteins in common for DONtreated cells (DNA topoisomerase 1 -P11387- and DNA topoisomerase 2-alpha -P11388-), and only one for ZEA-treated cells (chromobox protein homolog 5 -P45973-) (Table 2). In addition, the identified proteins common to the toxins alone and the combination after 1 h exposure were different to those shared after 24 h of exposure. Comparing the DON + ZEA-response proteins between 1 h and 24 h exposure, only three proteins were found in common: the ATP-binding cassette sub-family F member 1 (O8NE71), DNA topoisomerase 1 (P11387) and DNA topoisomerase 2-alpha (P11388) (Table 2). All these common proteins were at lower abundances than in the control treatment after both incubation periods.

3.2. Molecular functions and subcellular locations of DON- and/or ZEA response proteins

The main molecular functions and subcellular locations of each differentially abundant protein induced by DON, ZEA or DON + ZEA exposure were categorized by searching the Uniprot (http://www.uniprot.org) and Gene Ontology (http://geneontology.org/) databases and are illustrated in Figs. 2 and 3. It is important to note that the same protein can possess several molecular functions and can be localized in different subcellular parts.

Molecular function annotations of the mycotoxin-response proteins (Fig. 2) revealed that, after 1 h exposure, all 21 DON-response proteins had binding and/or catalytic activity. More specifically, proteins with a binding molecular function mostly targeted DNA, RNA and proteins (80%), while 60% of those with a catalytic activity were hydrolases. In addition, the subcellular location analysis (Fig. 3) showed that almost half of these 21 DON-affected proteins were cytoplasmic and about 43% were localized in the nucleus. For the 6 ZEA-response proteins, they all showed either a binding (67%) or a catalytic activity (33%), all with a different binding target or a different catalytic function. Moreover, they were primarily localized in the nucleus (50%) and to the mitochondrion (33%). Similarly to DON- and ZEA-response proteins, most of the 35 DON + ZEA-targeted proteins after 1 h exposure were annotated with a binding and/or a catalytic activity (> 90%). More specifically, > 60%of the proteins with a binding activity targeted DNA or RNA and almost 60% of those with a catalytic activity had a hydrolase activity. Furthermore, > 60% of the DON + ZEA-affected proteins were localized in the nucleus and 25% were cytoplasmic.

After 24 h exposure, the analysis of the molecular functions (Fig. 2) further showed that a large majority of the 55 DON-response proteins



Fig. 1. Venn diagrams of differentially abundant proteins in HepaRG cells after (A) 1 h and (B) 24 h treatment with DON and/or ZEA. Bold numbers represent the number of proteins exhibiting differential abundance compared to the control. Numbers for proteins exhibiting higher (\nearrow) and lower (\searrow) abundances are also provided.

(about 80%) had a binding and/or a catalytic activity. In particular, most of those with a binding activity targeted nucleic acids (41%) and proteins (24%), while those with a catalytic activity mainly belonged to the hydrolase family (about 50%). In addition, the analysis of the subcellular locations (Fig. 3) showed that 38% of the DON-affected proteins were nucleic, 36% were cytoplasmic and 22% were localized in the cell membrane. Concerning the 96 ZEA-response proteins after 24 h exposure, they mostly presented a binding or a catalytic activity (74%) with several proteins exhibiting a nucleic acid or protein binding activity (38%) or a hydrolase activity (55%). Moreover, 40% of these

96 targeted proteins were localized in the nucleus, the same proportion was cytoplasmic and only 18% were mitochondrial. Regarding the 39 DON + ZEA-affected proteins, most of them had binding and/or catalytic activity (70%) after 24 h exposure. > 60% of the proteins had a binding activity targeting DNA or RNA and almost 60% of those with catalytic activity were hydrolases. Furthermore, after 24 h exposure, 31% of the DON + ZEA-response proteins were localized in the nucleus, 31% in the endoplasmic reticulum (ER) and 28% in the cytoplasm.

The molecular function analysis (Fig. 2) revealed that the remainder



Fig. 2. Annotated molecular functions of the response proteins in HepaRG cells after 1 h and 24 h exposure to DON, ZEA and DON + ZEA. Results are expressed as the percentage of affected proteins with the considered molecular function among all the affected proteins for each treatment.



Fig. 3. Annotated subcellular locations of the response proteins in HepaRG cells after 1 h and 24 h exposure to DON, ZEA and DON + ZEA. Results are expressed as the percentage of affected proteins with the considered subcellular location among all the affected proteins for each treatment.

of the mycotoxin-response proteins after both 1 and 24 h exposures had elongation factor, receptor, structural, transporter and/or translation regulator activity. Based on the definition of "catalytic activity" given by the Gene Ontology database, these first results indicated that the DON and ZEA mycotoxins primarily affected enzymes that targeted nucleic acids and proteins in HepaRG cells. Furthermore, for all treatments, the analysis of the subcellular location annotations revealed that a minor part of the affected proteins by these mycotoxins, alone or in mixture, were localized in organelles such as Golgi apparatus, endosome, peroxisome and lysosome, or to the cell junction and some others were secreted by the cells. The results suggested that DON might induce nucleic and cytoplasmic changes in human hepatocytes, while ZEA might also induce mitochondrial changes. When present together, these two mycotoxins mostly targeted the nucleus after 1 h exposure as well as the ER after 24 h exposure.

3.3. Analysis of the biological process annotations of DON- and/or ZEA response proteins

Study of the mycotoxin-response proteins with the functional annotation tool DAVID provided information about the main biological processes affected by DON and ZEA alone and in combination in human hepatic cells. Only biological process categories containing at least two proteins and with a *P* value < 0.05 were retained (Supplementary data, Table S2).

After 1 h exposure, DON induced significant abundance changes for proteins involved in 9 biological processes, the most represented ones being cell division, chromosome segregation and protein modification by small protein conjugation, with 5 proteins involved in each (corresponding to 24% of the 21 DON-response proteins). Regarding the 6 ZEA affected proteins after 1 h exposure, no enriched biological processes were identified by DAVID. For the DON + ZEA condition, more biological processes were affected than DON and ZEA alone (n = 27) and targeted chromosome organization, DNA metabolic process and cell cycle involving 13 (37% of response proteins), 12 (34%) and 10 (31%) proteins, respectively. In addition, 6 biological processes

constituted a common response between differentially abundant proteins after 1 h exposure to DON and 1 h exposure to DON + ZEA (chromosome segregation, DNA topological change, embryonic cleavage, meiotic chromosome separation, protein sumoylation (*i.e.* attachment of a small ubiquitin-like modifier) and resolution of meiotic recombination intermediates), with the same proteins contributing in each case.

After 24 h exposure, 47 biological processes appeared to be affected by the DON exposure. The most represented ones were involved in transport and adhesion, such as vesicle-mediated transport (n = 17proteins), cell adhesion (n = 14), biological adhesion (n = 14) and transport regulation (n = 13) (corresponding to 31%, 25% and 24% of the 55 DON-regulated proteins, respectively). In addition, 11 proteins (i.e. 20%) were also involved in cell development and cell proliferation. Regarding ZEA, 30 biological processes appeared to be affected, the most important ones being cellular metabolic process (n = 25 proteins), cellular response to stress (n = 22) and cell proliferation (n = 16) (representing 26%, 23% and 17% of the 96 ZEA-regulated proteins respectively). Moreover, several biological processes involved in the cellular response to numerous compounds including drugs seemed to be impacted following 24 h exposure to ZEA (about 10 biological processes from 21 proteins). For the DON + ZEA mixture, after 24 h exposure, 31 biological processes were identified. Most of the DON + ZEA-response proteins after 24 h exposure were involved in gene expression (n = 16) and hydrolase activity (n = 10) regulations (corresponding to 41% and 26% of the 39 DON + ZEA-regulated proteins respectively). Furthermore, after 24 h exposure, 12 biological processes were common to DON and DON + ZEA (apoptotic cell clearance, regulation of apoptotic cell clearance, positive regulation of apoptotic cell clearance, blood vessel development, blood vessel morphogenesis, extracellular matrix organization, extracellular structure organization, phagocytosis, regulation of phagocytosis, positive regulation of phagocytosis, positive regulation of endocytosis, and positive regulation of transport) while only 1 biological process (phagocytosis) was common to ZEA and DON + ZEA (which was common to DON too).

Measured concentrations of DON and ZEA (μ M) in the cell supernatants after 0 h, 1 h and 24 h of exposure with HepaRG cells (mean \pm SD of concentrations from 3 independent experiments quantified using LC-MS). * = Mean of the final concentration significantly different from the initial concentration (P < 0.05); N.D. = not detectable.

Culture condition	Control culture	DON-treated culture	ZEA-treated culture	DON + ZEA-treated culture
Initial concentration of DON (µM)	ND	0.20 ± 0.0	ND	0.20 ± 0.0
Final concentration of DON after 1 h exposure (µM)	ND	0.26 ± 0.0	ND	0.23 ± 0.1
Final concentration of DON after 24 h exposure (µM)	ND	0.27 ± 0.4	ND	0.24 ± 0.4
Initial concentration of ZEA (µM)	ND	ND	20.0 ± 0.0	20.0 ± 0.0
Final concentration of ZEA after 1 h exposure (µM)	ND	ND	21.4 ± 0.6	20.2 ± 0.8
Final concentration of ZEA after 24 h exposure (μM)	ND	ND	$0.03 \pm 0^{*}$	$0.06 \pm 0^{*}$

A comparison of the affected biological processes after 1 h and 24 h of treatment showed that, for DON, none were common between both exposure durations while, regarding the mycotoxin combination, only embryonic cleavage was in common.

3.4. Measurement of DON and ZEA concentrations in cell supernatants after 1 h and 24 h exposure

In order to quantitatively evaluate the DON and ZEA uptake by the HepaRG cells after 1 h and 24 h exposures, concentrations of these two fusariotoxins were determined in cell supernatants at the end of the incubation periods and compared to the initial concentration used (namely IC₁₀). Based on the standard curves (linearity (R^2) > 0.99; data not shown), mycotoxins were quantified in cell supernatants. After 1 h exposure, no significant difference was observed between the initial concentrations and the final concentrations of DON and ZEA in both the mono- and co-exposure conditions (Table 3). After 24 h exposure, a reduction in ZEA was observed as concentrations decreased from 20 μ M to 0.03 and 0.06 μ M in supernatants from cells exposed to ZEA alone and those treated with DON + ZEA, respectively, whereas no difference was observed for DON (Table 3).

4. Discussion

Currently, there is a strong demand for better assessment of health risks related to multiple mycotoxin exposures, as well as to low concentration exposures on short and long time frames using relevant, appropriate models. In addition, in the field of toxicology, certain hypotheses need to be confirmed or discarded, including the endocrine disrupting effect of some mycotoxins (such as ZEA) and their carcinogenic potential (such as TCTs and ZEA) as the International Agency for Research on Cancer (IARC) classified these mycotoxins in group 3 (i.e. unclassifiable as to carcinogenicity in Humans) due to the lack of data [22]. Furthermore, the discovery of exposure or effect bioindicators using high-throughput methods (transcriptomics, proteomics, metabolomics) is strongly expected to offer new arguments for epidemiological assessment of the risks incurred by consumers [23]. In this context, using proliferating HepaRG cells, which are recognized as a pertinent model for toxicological studies [24], we investigated the underlying toxicity mechanisms for low doses of DON and ZEA at the proteome level. The effect of the DON + ZEA mixture was also studied in the same conditions since the toxicity of mycotoxins in a mixture cannot always be predicted based upon their individual toxicities [3,25]. More specifically, we characterized the early proteomic changes (after only 1 h exposure) associated with low-dose exposure to DON and/or ZEA using LC-MS/MS analyses, and we compared the obtained candidate mechanism-based proteins to those identified after 24 h exposure using the same doses.

The first aim of this study was to choose, for each fusariotoxin, a dose that would induce a significant cellular response that can be easily observed by proteomics while limiting cell mortality in order to protect proteomic analyses against events that are strictly related to cell death. Achieving cellular death would not provide relevant insights into the specific molecular mechanisms involved in toxic injury. Therefore, in the context of this study, we used 0.2 and 20 μM for DON and ZEA, respectively, for proteomic experiments and verified that no cytotoxicity was induced on HepaRG cells at these concentrations after 24 h exposure (data not shown). It should be noted that these concentrations are higher than the ones estimated in human blood. For example, Maresca [26] estimated DON concentration to be 1.5 nM in human blood. This estimation was based on the provisional maximum tolerable daily intake (PMTDI) of 1 µg of DON/kg of bw/day, and assuming that toxicokinetics data obtained with pigs orally exposed to DON could be extrapolated to humans. Similarly, Shin et al. [27] predicted ZEA concentration in human blood from a physiologically based pharmacokinetic (PBPK) model for ZEA following oral dosing in rats. The authors calculated that an exposure of 0.0312 mg of ZEA/kg of bw/day was necessary to have a steady-state ZEA concentration in human blood of 0.014 ng/ml. This means that, for a PMTDI of $0.2 \mu g$ of ZEA/kg of bw/day, the ZEA blood concentration should be about 280 nM. Furthermore, it has been reported that patients with serum levels of 19-100 g/ml ZEA exhibited an increasing incidence of early thelarche [28]. Noteworthy, as stated, these values in human blood were obtained from PMTDI which may be exceeded according to the ingested quantities of contaminated product. The tested concentrations corresponded to the IC₁₀ concentrations determined in a previous study after 48 h exposure [11].

Then, after cell exposures of 1 h and 24 h with DON and ZEA alone and in combination, we performed the LC-MS/MS analyses. After only 1 h exposure to mycotoxins, we observed a small but significant change in the proteomes. By comparison, after 24 h exposure to the mycotoxins, about 3 times more proteins were impacted. Furthermore, very few proteins were shared between the individual and combined exposures as well as between the two exposure durations for a same mycotoxin or the mixture, making it difficult to identify potential exposure markers. This issue is also supported by the fact that, among all the mycotoxin-response proteins, very few proteins were at higher abundances than in the control and none appeared to be secreted. However, our results highlighted 2 proteins at significantly lower abundances in all treatments (except after 1 h ZEA incubation): DNA topoisomerase 1 (P11387) and DNA topoisomerase 2-alpha (P11388). Although these enzymes have been already shown to be inhibited by several compounds including the ochratoxin A mycotoxin [29] and some Alternaria mycotoxins [30], these results pointed out an important cellular mechanism linked to DON and ZEA toxicity. Thus, they were not considered as specific response proteins to DON or ZEA, and could rather be part of a generic mycotoxin response system or generic immune system response. Our results also showed that DON and ZEA generated very different proteomic profile changes, alone and in combination, depending on the exposure period. Another noticeable point was that DON induced more proteomic changes than ZEA in HepaRG cells after 1 h exposure, whereas the opposite was observed after 24 h exposure. Furthermore, more proteins were differentially abundant due to the mycotoxin mixture than to the toxins alone after 1 h treatment while the contrary was observed after 24 h exposure. These latter findings might suggest a synergistic or additive effect of the mixture on the proteome of HepaRG cells after 1 h exposure, and an antagonistic effect after 24 h exposure. In a previous study, targeting certain liver-
specific functions, we also observed synergism for the DON + ZEA combination at the gene level on HepaRG cells after 1 h exposure using the same concentrations as in the present study [11].

Analysis of the molecular function and subcellular location annotations was in agreement with the literature indicating that DON is a translational inhibitor that binds to eukaryotic ribosomes and thus inhibits DNA, RNA and protein synthesis [31,32] and may cause impairment of membrane function [33]. On the other hand, ZEA has been reported to mainly target mitochondria and/or lysosomes [34,35]. Nevertheless, in our study, no changes in lysosomes were noticed after ZEA exposure on HepaRG cells whereas important changes in the nucleus were observed. When present together, DON and ZEA appeared to affect, the endoplasmic reticulum (ER) after 24 h exposure. A recent review from Rabilloud et Lescuyer [36] reported that the emerging keywords from several analyses of toxicoproteomic responses to natural products such as drugs are "ER stress" and "mitochondrial responses", which suggests they are core cellular responses to which many different toxin mechanisms converge. As explained in this review, the ER and mitochondrial stress response are commonly activated by toxicants due to i) the ability of the mitochondria to pump various cationic species that become concentrated within the mitochondrial matrix and to ii) the fact that ER is the site of localization of major metabolizing enzymes such as cytochromes P450, which degrade organic toxicants but may release more toxic products. Interestingly, our results showed that the 4F22 cytochrome P450 (Q6NT55) was one of the proteins affected by ZEA and DON + ZEA after 24 h exposure.

Analysis of the biological process annotations using UniProt and Gene Ontology databases (data not shown) revealed that the DON and ZEA mycotoxins did not initiate a massive stress response in HepaRG cells after 1 h exposure when present individually, as seen by the limited number of differentially abundant stress protein (4 proteins for DON and 3 proteins for ZEA). The DON + ZEA combined 1 h exposure induced more proteomic changes in the human hepatocytes than their individual exposures by, for instance, slightly exacerbating the cell response to stress. A total of 9 proteins involved in the cellular stress response were affected by the mycotoxin combination after 1 h exposure. However, after 24 h exposure, the opposite effect was observed: the individual exposures of DON and ZEA seemed to induce a higher cellular stress response than their combined exposure (20 and 36 proteins with a stress response biological function for DON and ZEA respectively and only 12 proteins for the mixture). In addition, both DON and ZEA significantly reduced the abundance of the multidrug resistance protein 1 (P08183) - that is responsible for decreased drug accumulation in multidrug-resistant cells - after 24 h exposure while the mixture did not. Thus, these results indicated a possible mitigation or protection strategy utilized by the hepatocytes when exposed to the mycotoxin cocktail for long periods of time. This aspect needs further study to better understand what type of regulation is implicated by the cells when they are facing a mycotoxin cocktail. However, despite the very low mycotoxin doses applied in the present study, all treatment conditions (with the exception of ZEA after 1 h exposure) seemed to induce important pathways related to programmed cell death by affecting the abundances of proteins involved in MAPK signaling pathway (including, for example, the 26S proteasome non-ATPase regulatory subunit 8 - P48556 - for DON 1 h, integrin alpha-V - P06756 - and rasrelated protein Rap-1A - P62834 - for DON, ZEA and DON + ZEA 24 h or mitogen-activated protein kinase 1 - P28482 - and mitogen-activated protein kinase 3 - P27361 - for ZEA 24 h). Recently, two studies highlighted the relationship between protein phosphorylation and DON immunotoxic effects [37,38], suggesting the need for further studies using phosphoproteomic techniques.

Analysis of the main biological processes by the functional annotation tool DAVID for DON after 1 h exposure was in accordance with both the analysis of the molecular function and location annotations and with the literature (indicating that DON inhibits DNA, RNA and protein synthesis [31,32]). In addition, the obtained results suggested an effect of DON on protein (post) translational modifications, which was in accordance with the study of Graziani et al. [39] reporting that DON affects iNOs protein expression in human enterocytes through an increase in its ubiquitinylation and degradation by the proteasome. After 24 h of DON exposure, more biological processes were affected (and were mostly related to cell adhesion and transport), but none were common to those impacted after 1 h suggesting a cascade of cellular events along this 24 h period. These results seemed to be correlated with the analysis of the subcellular location annotations (highlighting that the cell membrane was one of the main target of DON after 24 h). After 24 h exposure, ZEA was the treatment that induced the most proteomic changes in HepaRG cells, mainly targeting proteins involved in the cellular response to stress. For the mycotoxin mixture, by comparison with the individual exposures, the DON + ZEA-impacted biological processes were more numerous after 1 h and less numerous after 24 h, but in both cases, most of them appeared to be different than for DON or ZEA single exposures. In addition, as for the individual exposures, the DON + ZEA affected biological processes were very different between 1 h and 24 h. Using transcriptomic and proteomic approaches targeting some hepatic-specific functions, we previously noticed that the cellular response of HepaRG cells to acute (1 h) and chronic (14 and 28 days) exposures to DON and/or ZEA was very different depending on the incubation period, doses and the considered mycotoxin or mixture [40,41].

Despite this significant diversity in the effects, it is interesting to notice that some biological processes affected by DON single exposure were also impacted by the mixture after both exposure periods while only one of those affected by ZEA individual exposure was also impacted by the combination after 24 h exposure (and 0 after 1 h). Thus, these results suggested that, when DON and ZEA were present together, DON might inhibit some toxic effects of ZEA while impacting new biological pathways, specific to the mixture. The same trends were observed at the metabolome level by Ji et al. [42]. These authors explored the endo- and exo-metabolomes of murine macrophages (ANA-1 cells) after 24 h exposure to DON and ZEA alone and in combination and reported that DON, when present simultaneously with ZEA, may inhibit certain toxic effects of ZEA (namely the estrogenic effects), and that new pathways appeared to be affected by the mixture compared to the toxins alone (namely palmitic acid metabolism). Nevertheless, their results showed that amino acid metabolism and glycometabolism were the two dominant pathways affected by the three conditions. Here, even if after 24 h exposure, all treatment conditions altered the abundances of some proteins involved in the metabolism of various amino acids (as observed from the provided biological processes for each protein using the UniProt database, data not shown), no amino acid biological pathways were significantly affected by the mycotoxins (as observed with DAVID, Table S2). Overall, in this study, we mainly observed a high impact of the mycotoxins and the mycotoxin mixture on cell cycle, cell proliferation and/or cell development, as well as on DNA metabolic processes. However, for DON alone, which is known to induce inflammation and upregulation of several cytokines in numerous cell models [43-45], no significant inflammatory response was highlighted by the biological process analysis using DAVID. This result might suggest that the pro-inflammatory effect of DON can only be observed at higher doses than the one used in our study (0.2 μ M). Noteworthy, the DON + ZEA mixture induced such a response after 24 h exposure (Table S2). In addition, the estrogenic effect of ZEA is commonly described in the literature [34,42] but it is rarely reported for DON. Nonetheless, in the present study, we observed a reduction in estradiol 17-beta-dehydrogenase 2 (P37059) abundance - which is an enzyme capable of catalyzing the interconversion of testosterone and androstenedione, as well as estradiol and estrone, following DON exposure (after 1 h) but not after ZEA exposure. In vivo, some authors reported reproduction impairments induced by DON on animals, such as decrease in litter size and increase in postnatal mortality [4-6]. Furthermore, we also observed that DON and the DON + ZEA mixture affected 19 and 15 proteins involved in cancer after 24 h exposure respectively (P value < 0.05). It would be interesting to verify if this effect is maintained at longer incubation times and leads to a cancer phenotype.

Finally, since we observed significant effects of the toxins on HepaRG cells, mainly on the nucleus from 1 h exposure, we wondered if this was due to a fast mycotoxin uptake by the cells. Mycotoxin quantification by LC-MS/QTOF in the cell medium revealed no changes in ZEA or DON concentrations after 1 h exposure at IC10 while proteomic changes were induced in HepaRG cells. Even more interestingly, after 24 h exposure, no changes in DON concentration were observed neither for DON alone nor for the DON + ZEA mixture. These observations raised the question about the activation mechanism of the cellular response. In this context, some hypotheses can be proposed, i) nonquantifiable trace amounts of the tested mycotoxins using the selected method (detection limit 0.01 μ M using the [M-Na] + 319.115 m/z ion in ESI + mode) can enter the cell suggesting high cell sensitivity or ii) binding of the mycotoxins to membrane receptors, as already reported by Maresca [26], or iii) a combination of both mechanisms. The important number of proteins regulated by ZEA after 24 h exposure could be explained by the much lower measured concentration of ZEA in the cell supernatants at the end of this incubation period. This result suggests that ZEA would be easily absorbed or metabolized by the hepatocytes, while DON would not be. Noteworthy, while DON has been shown to enter numerous animal/human cell types, Königs et al. showed that DON is metabolized neither by human primary hepatocytes nor HepG2 cell line [46]. As the HepaRG cell line was used in this study, the observed results may be associated with the studied cell type and suggest that proliferating HepaRG cells do not metabolize DON. These results might be partly explained by the higher log P (or log K_{OW}) value of ZEA compared to DON (namely 3.58 for ZEA and -0.71 for DON), indicating that ZEA is much more lipophilic than DON and thus can more easily enter the cell membrane.

In humans, very little data are available on the toxicokinetics of DON and ZEA. The major characterized metabolite of DON, de-epoxy DON (DOM-1), is usually found in urine and stools of animals exposed to DON [47] and is produced via intestinal or rumen microbiota activity rather than by the liver [32]. Pestka et Smolinski [33] reported that, in humans, drug-metabolizing enzymes such as CYP450 enzymes do not detoxify DON into DOM-1. However, in the liver, DON can be conjugated to glucuronides leading to the formation of a non-toxic detoxification product, glucuronide conjugated-DON (DON-GLU) [48]. Only unmetabolized DON as well as DON-GLU were detected in human urine sample analyses [49]. Furthermore, the liver, as the small intestine, were also reported to be one of the main sites of deacetylation of DONacetylated derivatives present in cereals (i.e. 3-acetyl and 15-acetyldeoxynivalenol), resulting in the generation of DON [50]. Concerning ZEA, human intestinal microflora cultured in a continuous flow system were unable to degrade this mycotoxin [28]. In vitro, the α -zearalenol metabolite has been described as the most preponderant metabolite followed by β -zearalenol using the human intestinal epithelial cells, Caco-2 [51]. Additional data on ZEA metabolites in the cell medium and in the intracellular fraction could verify if the cells absorbed or metabolized this mycotoxin after 24 h exposure since the abundance of the glutathione S-transferase A3 (Q16772) following 24 h to ZEA exposure was modified (Table S1). Indeed, this enzyme conjugates toxicants to a glutathione molecule to prevent its binding to the target. In a previous study, at very low cytotoxic doses (corresponding to the maximum level permitted by the European regulation in cereals intended for direct human consumption), we observed high cytotoxicity of DON alone and in a mixture with ZEA on HepaRG cells in chronic exposure conditions (i.e. cells treated with the mycotoxins every two days over 14 days), while ZEA alone didn't induce cell mortality [41]. This could be explained as stated above by the fact that DON would not be metabolized or neutralized by the proliferating HepaRG cells, contrary to ZEA.

5. Conclusion

In this study, proteomic mechanisms underlying the observed cytotoxic effects of low doses of DON and ZEA in proliferating HepaRG cells were revealed using tandem mass spectrometry. The results showed that, despite the diversity of cellular mechanisms involved in the response to the mycotoxins alone and in combination, some similar proteins and thus biological processes were shared between DON alone and the combined DON + ZEA treatment, while no similarities were observed between ZEA-treated cells and DON + ZEA-treated cells. Moreover, these results also revealed that human hepatic cells seem to be very sensitive to DON and ZEA, as highlighted by the observed proteomic changes after only 1 h exposure to low mycotoxin doses thus confirming that very low doses were able to impair cellular homeostasis. These findings also showed that different cellular pathways responded to the different single and combined mycotoxins and to the different incubation periods, emphasizing the need to further explore the regulation capacities of the cells with more complex incubation kinetics and by combining "omics" tools. This innovative, combined analysis of the toxicity, global proteome changes and mycotoxin quantification has specifically revealed that DON was able to induce toxicity in acute conditions affecting the cell at the proteomic level while it was not apparently absorbed by the proliferating HepaRG cells. In conclusion, this raises the question as to whether a DON-sensing mechanism exists and should be further investigated.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Transparency document

The http://dx.doi.org/10.1016/j.jprot.2017.11.025 associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2017.11.025.

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Les points marquants de cette étude sont les suivants :

- Les hépatoblastes humains semblent être très réactifs à l'exposition au DON et à la ZEA, comme le montrent les changements protéomiques observés après seulement 1h d'exposition à de très faibles doses. En effet, le niveau d'abondance de 6 protéines varie après l'exposition à la ZEA, et celui de plus d'une vingtaine de protéines est modifié après l'exposition au DON.
- Selon la période d'incubation et la mycotoxine ou le mélange considéré, la réponse cellulaire apparaît très différente. En effet, très peu de protéines dont l'abondance varie sont communes aux différents traitements.
- De manière générale, les principaux processus biologiques affectés par ces différents traitements sont le cycle cellulaire, la prolifération cellulaire et/ou le développement cellulaire, ainsi que le processus métabolique de l'ADN.
- Le mélange de mycotoxines semble induire d'avantage de changements au niveau du protéome des cellules par rapport aux mycotoxines seules après 1h d'exposition, alors que le contraire est observé après 24h. En particulier, l'importante réponse cellulaire au stress induite par l'exposition à la ZEA après 24h semble être diminuée en présence de DON.
- Alors qu'une réponse cellulaire au niveau protéomique est observée pour les deux mycotoxines testées, les dosages de mycotoxines résiduelles dans les surnageants de culture montrent que seules des traces de ZEA sont retrouvées après 24h, alors qu'aucune différence avec le contrôle n'est observée pour le DON. Ces éléments interrogent sur l'existence possible de mécanismes senseurs de la présence des mycotoxines (en particulier DON) dans l'environnement de la cellule.

La figure 10 schématise de façon synthétique les principaux résultats obtenus dans cette étude.



Figure 10 : Représentation schématique des principaux effets biologiques observés dans l'étude n°3.

Partie III : Evaluation de l'hépatotoxicité chronique du déoxynivalénol et de la zéaralènone seuls et en mélange sur les cellules HepaRG

Etude n°4:

Individual and combined toxicological effects of deoxynivalenol and zearalenone on human hepatocytes in *in vitro* chronic exposure conditions

Toxicology Letters

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Marie-Caroline Smith, Stéphanie Madec, Audrey Pawtowski, Emmanuel Coton, Nolwenn Hymery

L'objectif de cette troisième partie était d'étudier les effets de l'exposition et de la coexposition au DON et à la ZEA sur les cellules humaines d'origine hépatique suite à une exposition chronique, c'est-à-dire après une exposition répétée pendant plusieurs semaines à des doses non cytotoxiques. En effet, cette démarche est plus proche de la réalité de l'exposition de l'Homme aux mycotoxines *via* son alimentation, qu'une exposition aiguë à ces toxines. De plus, les données *in vitro* en chronique sont, à l'heure actuelle, quasi-inexistantes. Les cellules HepaRG, qui conservent de façon stable et élevée des activités spécifiques du foie pendant plusieurs semaines, semblent donc particulièrement adaptées pour ce type d'étude (Aninat et al., 2006; Guillouzo et al., 2007).

Plus particulièrement, les HepaRG ont été exposées pendant 14, 28 et 42 jours à différentes concentrations sub-toxiques du DON et de la ZEA, à savoir : i) la dose moyenne (DM) d'exposition aux mycotoxines des Français à l'âge adulte déterminée par Leblanc et al. (2005), ii) la dose maximale journalière tolérable (DJT) établie par le JECFA (Joint Expert Committee for Food Additives) (JECFA, 2002) et iii) la teneur maximale (TM) autorisée par la réglementation européenne dans les céréales destinées à l'alimentation humaine directe (EC N° 1881/2006).

Les effets du DON, de la ZEA ainsi que du couple DON+ZEA ont donc été évalués pour ces trois périodes d'exposition et pour ces trois concentrations sur i) la viabilité cellulaire, *via* un

test MTS, ii) l'expression des gènes codant pour les facteurs spécifiques des hépatocytes précédemment étudiés dans la partie II (à savoir CYP3A4, CYP4F3B, C\EBP α , HNF4 α , aldolase β , transferrine et albumine), par qPCR ainsi que sur iii) l'expression au niveau protéique de quelques-uns de ces facteurs (en particulier, CYP3A4 et albumine) et de la claudine-1 par Western blot.

Il est à noter que, dans cette étude, après plusieurs semaines à confluence, les cellules HepaRG se sont différenciées. Ainsi, deux populations cellulaires sont distinguables : des hépatocytes et des cellules épithéliales biliaires, qui n'ont pas été séparées dans notre étude.

La figure 11 schématise de façon synthétique les principaux effets biologiques évalués dans cette étude.



Figure 11 : Résumé schématique des différents effets biologiques et conditions évalués dans l'étude n°4.

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Individual and combined toxicological effects of deoxynivalenol and zearalenone on human hepatocytes in *in vitro* chronic exposure conditions



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ABSTRACT

While numerous surveys highlighted the natural co-occurrence of mycotoxins in food, data about their toxicological combined effects is still limited. This is especially the case for chronic exposure conditions, although the latter are more representative of the mycotoxin risk associated with food consumption than acute exposure. In the present study, cell viability and gene expression levels of relevant hepatocyte-specific functions were evaluated for the HepaRG human liver cell line exposed to deoxynivalenol (DON) and/or zearalenone (ZEA) during 14, 28 and 42 days at three subtoxic concentrations corresponding to i) the determined average exposure dose of French adult population, ii) the tolerable daily intake established by the Joint FAO/WHO Expert Committee and iii) the maximum level permitted by the European regulation in cereals intended for direct human consumption. For the latter, DON and DON + ZEA induced 90% cell mortality after 14 days. In addition, depending on the considered toxin or mixture, doses and exposure periods, important variations of gene expression levels were observed. Despite the fact that *in vitro* conditions differ from the *in vivo* situation, the obtained results clearly highlighted that long-term toxicological effects of chronic exposure to mycotoxin combinations should be further investigated and, if necessary, taken into consideration at the regulatory level.

1. Introduction

Among food contaminants, mycotoxins are particularly important from the safety and economic points of view. Mycotoxins are natural compounds produced by various fungal species (such as *Aspergillus, Penicillium, Fusarium, Alternaria,* and *Claviceps* spp.) and are found all around the world as natural contaminants, mostly in commodities of plant origin (Bennett and Klich, 2003). Mycotoxin production, especially on grains, is highly dependent on pre and/or postharvest environmental factors, climate being the key factor in both fungal and mycotoxin occurrence. Thus, fungal infections and mycotoxin production are very hard to avoid and control (Milani, 2013). Nowadays, natural co-occurrence of mycotoxins in commodities is well-established as shown by a three-year worldwide survey reporting that 48% of 7049 analyzed feedstuffs samples were contaminated by two or more mycotoxins (Rodrigues and Naehrer, 2012). This co-occurrence can be explained by at least three reasons: i) most fungi can produce several mycotoxins concurrently, ii) food commodities can be contaminated by several fungi simultaneously or in a quick succession and iii) diets usually correspond to a combination of various food commodities. Among the infinite number of possible encountered mycotoxin mixtures, combinations of toxins from *Fusarium* spp. (called fusariotoxins) are of primary concern because of their high occurrence in the North temperate zone of the world. In particular, deoxynivalenol (DON) in combination with zearalenone (ZEA) is one of the most widespread fusariotoxin mixtures in grains (Smith et al., 2016). Furthermore, some authors have described additive and/or synergistic cytotoxic effects of DON + ZEA combination, highlighting a significant threat to human and animal health (Ficheux et al., 2012; Kouadio et al., 2007; Wan et al., 2013).

Trichothecenes (TCT) correspond to an important fusariotoxin family which may be divided in 4 groups according to their physical and chemical properties (A, B, C and D groups). Exposure to TCT may lead to apoptosis in several organs including lymphoid organs,

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Abbreviations: AED, Average exposure dose of French adult population; DMSO, Dimethylsulfoxide; DON, Deoxynivalenol; EC, European Commission; IC, Inhibitory concentration; JECFA, Joint Expert Committee for Food Additives; ML, Maximum level permitted in cereals by the European regulation; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethox-yphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); RT-qPCR, Real-time quantitative polymerase chain reaction; TBS, Tris-buffered saline; TCT, Trichothecenes; TDI, Tolerable daily intake established by the JECFA; ZEA, Zearalenone

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hematopoietic tissues, liver, intestinal crypts, bone marrow and thymus (Yazar and Omurtag, 2008). At the molecular level, TCT have been shown to be potent inhibitors of protein synthesis, DNA and RNA synthesis and mitochondrial function in eukaryotes (Bezerra da Rocha et al., 2014; Rocha et al., 2005). DON, which belongs to type B TCT, appears to be one of the most widespread mycotoxins in grains, although it is less toxic than other major TCT. DON consumption mainly causes food refusal, nausea, vomiting and diarrhea at high acute doses, while longer exposure results in weight loss, reduced growth and adverse effects on the thymus, spleen, heart and liver (Bosco and Molle, 2012; Pestka and Smolinski, 2005; Sobrova et al., 2010). In the liver, DON can be conjugated to glucuronides (Gareis et al., 1987) leading to the formation of DON glucuronide (DONGLU), a non-toxic detoxification product as shown by Wu et al. (2007). Furthermore, the liver was also reported to be one of the main site of deacetylation of DONacetylated derivatives present in cereals (i.e. 3-acetyl and 15-acetyldeoxynivalenol), resulting in the generation of DON (Ajandouz et al., 2016). Concerning ZEA, this non-steroidal estrogenic mycotoxin has a similar structure to the 17β-estradiol endogenous estrogen. ZEA is usually non-lethal but it produces estrogen gene activation and causes functional and morphological alterations on reproductive organs (Tatay et al., 2014; Zinedine et al., 2006). Moreover, ZEA is mainly metabolized in the liver where it was found to be hepatotoxic by inducing adverse liver lesions with subsequent development of hepatocarcinoma (Hassen et al., 2007; Wang et al., 2014).

In the past, several episodes of severe mycotoxicosis in humans and animals due to the consumption of diets contaminated with high doses of mycotoxins have been reported. One of the most well-documented disorders in which TCT were possibly involved is referred to as "Alimentary Toxic Aleukia" (ATA). This situation, associated with episodes of lethal human disease in Russia, occurred during the 1940s and 1950s (Richard, 2007). Numerous papers reported that the human population is exposed to low doses of mycotoxin through food consumption. For instance, Roscoe et al. (2008) demonstrated regular occurrence of low levels of multiple mycotoxins in breakfast cereals on the Canadian market from a 3-year period survey, DON being the most frequently detected mycotoxin. More recently, Sundheim et al. (2017) reported that, in Norway, the estimated dietary DON intakes in adolescent and adult populations are in the range of the tolerable daily intake (TDI) or below. In France, Leblanc et al. (2005) calculated the average and high percentile intakes for major mycotoxins in adults, children and vegetarian populations. The results showed that the observed contaminant levels in ingested diets complied fully with current European legislation, even if specific population groups, such as children and vegans, could be exposed to DON and ZEA in quantities that exceed the tolerable or weekly daily intake levels, cereals and cereal products being the main contributors to high exposure. Noteworthy, the last French total diet study carried out between 2007 and 2009 by Sirot et al. (2013) showed that exposure to DON appeared to be of health concern for adult populations too. This highlighted the necessity to reduce the dietary exposure to DON and its metabolites which could be achieved by reducing the consumption and/or the maximal limits in food. Currently, very few data exist on chronic human mycotoxin exposure. In animals, a study on pig chronic exposure to fusariotoxins recently reported that DON, in combination with nivalenol or ZEA, induced a significant decrease in weight gain and increase in histological changes on liver (Gerez et al., 2015). Thus it appears that the effects of chronic exposure to mycotoxin multi-contamination is an important future challenge in human risk assessment.

Liver being our main detoxification organ, human hepatocytes represent one of the most relevant *in vitro* standard models for xenobiotic metabolism and toxicity studies. However, human primary hepatocytes are phenotypically unstable, have a limited life span and exhibit large inter-donor variability. On the other side, immortalized hepatic cell lines, such as HepG2, are devoid of substantial liver-specific functions, especially major cytochromes P450 which are involved in xenobiotic metabolism, and are consequently of limited interest (Guguen-Guillouzo and Guillouzo, 2010; Wu et al., 2016). However, the HepaRG human hepatoma cell line, derived from a human hepatocellular carcinoma, seems to be a surrogate of interest to primary human hepatocytes for toxicity studies. Indeed, HepaRG cells possess both the metabolic performances of primary hepatocytes and growth capacity of hepatic cell lines (Guillouzo et al., 2007). In particular, contrary to other human liver cell lines, HepaRG cells maintain many liver-specific functions, including expression of major cytochromes P450, membrane transporters, nuclear receptors and Phase II enzymes. In addition, HepaRG cells can be maintained for weeks with high and stable liver-specific activities (Aninat et al., 2006; Guillouzo et al., 2007).

The aim of the present study was to assess, in chronic exposure conditions, the single and combined effects of the DON and ZEA fusariotoxins towards the HepaRG human liver cell line. In this context, three subtoxic concentrations corresponding to i) the determined average exposure dose (AED) of French adult population (Schothorst and van Egmond, 2004), ii) the tolerable daily intake (TDI) established by the Joint Expert Committee for Food Additives (JECFA) and the Scientific Committee on Food (SCF) (JECFA, 2002) and iii) the maximum level (ML) permitted by the European regulation in cereals intended for direct human consumption (EC, 2006) were studied. Hepatotoxicity was evaluated through cell viability and gene expression of various liver-specific factors.

2. Materials and methods

2.1. Mycotoxins

DON standard (MW = 296.32 g/mol; CAS#51481-10-8) with purity > 98%, and ZEA standard (MW = 318.36 g/mol; CAS#17924-92-4) with purity > 99% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20 °C.

2.2. Cell and culture conditions

Human hepatocytes (HepaRG) were acquired from Biopredic International (Saint Grégoire, France). HepaRG cells were cultured in William's E medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 Units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin and 50 µM hydrocortisone hemisuccinate at 37 °C with 100% relative humidity (RH) in a 5% CO₂ atmosphere. Culture medium was replaced every 2 to 3 days with fresh growth medium. The adherent cells were passaged every 2 weeks at a density of 2.7×10^4 cells/cm² by a short time exposure (< 5 min) with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Sigma Aldrich), and reseeded in fresh medium.

To explore the individual and combined cytotoxic effects of DON and ZEA on HepaRG cells in chronic exposure conditions, 5×10^5 cells were treated with three subtoxic concentrations of DON and ZEA, corresponding to the AED, TDI and ML doses, respectively (Table 1) and were incubated at 37 °C in 24-well-plates during 14, 28 and 42 days. Culture medium was replaced every 2 days with fresh growth medium containing the tested mycotoxins. The final concentration of solvent in cell culture containing mycotoxins was not higher than 0.4%. Appropriate control cultures without mycotoxins but containing the same amount of solvent were included as negative controls. To convert the AED and TDI doses from µg of mycotoxin/kg of body weight/day to mol/l (or M), we considered that 1 kg of body weight equaled 11 of culture medium assuming that the recommended volume of culture medium by Biopredic International was established to deliver the appropriate quantity of nutriments for a related cell number by day (namely $12 \text{ ml}/10^6 \text{ cells}/2 \text{ days}$). In the same way, the ML doses were obtained by converting the established doses in μg of mycotoxin/kg of food to mol/l of culture medium. Thus, the established values (in μ g/kg Composition and relevance of each mycotoxin mixture.

Mixture	DON	ZEA	Relevance		
1 2 3	$\begin{array}{l} 1.6 \times 10^{-9} \text{M} \ (0.460 \mu\text{g/kg} \text{bw/d}) \\ 3.4 \times 10^{-9} \text{M} \ (1 \mu\text{g/kg} \text{bw/d}) \\ 2.5 \times 10^{-6} \text{M} \ (750 \mu\text{g/kg}) \end{array}$	$\begin{array}{l} 8.5\times10^{-11}M~(0.027~\mu g/kg~bw/d)\\ 6.3\times10^{-10}M~(0.2~\mu g/kg~bw/d)\\ 2.4\times10^{-7}M~(75~\mu g/kg) \end{array}$	Average exposure dose of French adult population (AED) Tolerable daily intake established by the JECFA (TDI) Maximum level permitted by the European regulation in cereals intended for direct human consumption (ML)		

bw/d for the AED and TDI doses or in μ g/kg for the ML doses) were divided by the related mycotoxin molecular weight (in g/mol) to obtain values in M.

For evaluation of CYP4F3B, CYP3A4, C/EBP α , HNF4 α , aldolase B, transferrin, albumin and claudin-1 encoding gene expression, 3×10^6 cells seeded in 25 cm²-flasks were exposed to DON and ZEA alone and in combination at the three selected subtoxic concentrations during 14 and 28 days.

2.3. Cytotoxicity evaluation by MTS assay

Mycotoxin cytotoxicity towards HepaRG cells was evaluated using the Promega CellTiter $96AQ_{\mathrm{ueous}}$ Non-Radioactive cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. This colorimetric method determines mitochondrial activity based on the reduction of a tetrazolium salt (MTS 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay) to formazan by mitochondrial dehydrogenases in viable cells. Briefly, at the end of the incubation period, culture media was removed and cells were washed with PBS. Then, 100 µl PBS were added in each well as well as 20 µL CellTiter 96AQueous Non-Radioactive solution and the cells were further incubated for 3 h. Formazan (MTS metabolite) was then quantified by reading the absorbance at 490 nm on a Multiskan FC plate reader (Thermo Scientific, Madison, WI, USA). Cell viability obtained for the negative control (cell cultures exposed to DMSO without mycotoxins) was defined as 100%. Cell viability mean percentages of three independent experiments \pm standard error of the mean (SEM) were used for statistical analyses.

2.4. RNA extraction and reverse transcription for cDNA synthesis

Total RNA was extracted from dried cell pellets using the RNeasy Protect Mini Kit from Qiagen (Valencia, Canada). RNA quantity and quality were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Madison, WI, USA). For cDNA synthesis, 1 μ g of total RNA was reverse transcribed for each sample using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystem, Foster City, CA, USA). Reverse transcription was performed as follows: 10 min at 25 °C, 2 h at 37 °C and 5 min at 85 °C using the peqSTAR 2× thermal cycler (PEQLAB – Life Science, VWR, Erlangen, Germany). The obtained cDNA was then stored at -80 °C.

2.5. Quantitative polymerase chain reaction for gene expression assessment

Quantitative polymerase chain reaction (qPCR) was performed by real-time fluorescent PCR using a C1000 thermal cycler with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). PCR primers designed by Madec et al. (2011) corresponding to TBP, Hs00427620_m1; CYP3A4, Hs00604506_m1; C/EBP α , Hs00269972_s1; HNF4 α , Hs00230853_m1; aldolase B, Hs01551887_m1; transferrin, Hs1067777_m1 and albumin, Hs00609477_m1 encoding genes were purchased from Life Technologies Ltd, Thermo Fisher Scientific (Paisley, UK) and used with the 5 x HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) for qPCR amplification. GAPDH (Forward-primer: CCCATCACCATCTTCCAGGAG; Reverse-primer: GTTGT-CATGGATGACCTTGGC) and CYP4F3 B (Madec et al., 2011) encoding genes were synthesized by Invitrogen, Thermo Fisher Scientific (Carlsbad, CA, USA) and used with the 5 x HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). For each transcript, a standard curve was constructed using the purified PCR product generated for each specific primer pair. Single reactions were prepared for each cDNA along with each serial dilution using the DNA-binding dye 5 x HOT FIREPol Probe qPCR Mix Plus or the 5 x HOT FIREPol EvaGreen qPCR Mix Plus from Solis BioDyne according to the manufacturer's recommendations. The cycling conditions for the 5 x HOT FIREPol Probe qPCR Mix Plus were 1 cycle of activation at 95 °C/15 min, followed by 40 three-segment cycles of denaturation at 95 °C/15 s and annealing/ elongation at 60 °C/60 s. Concerning the 5 x HOT FIREPol EvaGreen qPCR Mix Plus, the cycling conditions were as follows: 1 cycle of activation at 95 °C/15 min, followed by 40 three-segment cycles of denaturation at 95 °C/15 s, annealing at 62 °C/20 s and elongation at 72 °C/20 s. The baseline adjustment method of the Bio-Rad CFX Manager 2.1 software was used to determine the Ct in each reaction. All samples were amplified in triplicates and the mean value was used for further analysis. Relative gene expression levels were normalized to the GAPDH and TBP housekeeping gene expression and results were represented as fold change relative to the level expressed in untreated control cells (with solvent).

2.6. Western blot for protein expression measurement

HepaRG cells were lysed with 100 µl of ice-cold cell lysis buffer (NP40 Cell Lysis Buffer, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1 mM PMSF and protease inhibitor cocktail tablet (Complete EDTA-free, Roche Diagnostics, Mannheim, Germany). After 30 min on ice with vortexing at 10 min intervals, cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. The clear lysates were transferred in clean microfuges tubes and protein concentrations were determined using the colorimetric assay Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). For each sample, 20 µg of total protein were denatured by boiling at 99 °C for 10 min with a 1:4 dilution of 4 x Laemmli sample buffer (0.06 M Tris-HCl pH 6.8, 20% v/v SDS 10%, 10% v/v glycerol, 5% v/v bromophenol blue and 5% v/v β-mercaptoethanol in distilled water), completed with distilled water to 20 µl (final volume). Each sample was then entirely loaded onto a 12% polyacrylamide gel for protein detection and ran at 80 mA using a Mini-PROTEAN 3 Cell Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). Then, following the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, US), a transfer onto a nitrocellulose membrane (0.45 µm, GE Healthcare-Amersham, Buckinghamshire, UK) was conducted at 250 mA for 1 h. Membranes were saturated during 1 h with TBS (Tris-buffered saline)-0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) washing buffer containing 5% w/v nonfat dry milk. Membranes were probed overnight at 4 °C with appropriate primary antibody diluted in TBS-0.1% Tween 20 containing 5% BSA or nonfat dry milk (1:1000). Anti-active polyclonal CYP3A4, albumin, claudin-1 and β -actin (housekeeping protein) antibodies were purchased from Cell Signaling (Danvers, MA, USA) and stored at -20 °C. A horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in TBS-0.1% Tween 20 and 5% nonfat dry milk) (Cell Signaling, Danvers, MA, USA) was used for incubating membranes during 1 h. Band detection was performed with ECL Prime Western Blotting Detection Reagent (GE Healthcare-Amersham, Buckinghamshire, UK) and membranes were scanned using the G-Box system (Syngene, Cambridge, UK). Three independent experiments were performed and blots were analyzed using the Gene Tools analysis software (Syngene, Cambridge, UK). Relative protein expression levels were normalized to β -actin expression and results were represented as fold change relative to the level expressed in untreated control cells.

2.7. Statistical analysis

Data from cytotoxicity assessments as well as qPCR analyses were expressed as mean of three independent experiments \pm SEM. Statistical analyses were performed using the unpaired Student's *t*-test for comparison with control. Values of P < 0.05 were considered as significant.

3. Results

3.1. Effect of mycotoxin exposure on cell viability

Results from viability assessments showed that, even after 42 days of exposure, no cytotoxicity on HepaRG cells was observed at the AED and TDI doses for DON, ZEA and the DON + ZEA mixture (Fig. 1A and B). As for the AED and TDI doses, ZEA didn't show significant cytotoxicity at ML level even after 42 days of exposure (Fig. 1C). However, at the ML concentration for DON, about 90% of cell mortality was observed after 14 days of exposure and 100% after 28 days of exposure (Fig. 1C). The same effect as for DON alone was observed for the DON + ZEA mixture at the ML dose, thus revealing no antagonistic or synergistic effect of this mycotoxin combination on cell viability reduction.

3.2. Hepatocyte functional characteristics affected by mycotoxin chronic exposure

Expression levels of genes encoding for a set of liver-specific functions were measured for each treatment by RT-qPCR and results were reported in Table 2.

Interestingly, the results at the AED doses appeared very different

between 14 and 28 days of exposure. Indeed, after 14 days of exposure, results showed that the majority of tested genes were up-regulated while they were down-regulated after 28 days of exposure. More precisely, at 14 days, DON significantly increased the expression of two genes encoding for the HNF4a transcription factor and aldolase B glycolytic enzyme compared to the control condition. In comparison ZEA enhanced the expression of five genes encoding for the HNF4 α and C/ EBPa transcription factors, CYP3A4 drug-metabolizing enzyme as well as aldolase B and transferrin plasma protein. Regarding the DON-ZEA mixture, only the HNF4 α , CYP3A4 and transferrin gene expression was up-regulated. In addition, all the mycotoxin exposures reduced albumin gene expression. Interestingly, after 28 days of exposure, fewer genes seemed to be regulated by DON and ZEA individual exposures than after 14 days of exposure. Indeed, only a down-regulation of albumin gene expression was observed for DON, while ZEA only reduced albumin and C/EBPa gene expression, which suggests a time-dependent effect of these two mycotoxins on gene expression regulation. On the contrary, the DON + ZEA mixture induced several changes on the gene expression levels after 28 days of exposure, by highly down-regulating all studied hepatocyte-specific factor gene expressions.

At the TDI doses, the differences of gene expression between 14 and 28 days of exposure seemed to be less marked even if more up-regulations were shown at 28 days. At 14 days, DON single exposure increased CYP3A4 gene expression and down-regulated albumin gene expression, whereas ZEA up-regulated the expression of the CYP3A4, C/ EBPa and aldolase B genes, and reduced CYP4F3 B and albumin gene expression. Concerning the mycotoxin mixture, an increase in aldolase B gene expression and a down-regulation of albumin and transferrin gene expressions were observed. After 28 days, the expression variations were very different than after 14 days. DON up-regulated HNF4 α and aldolase B gene expression and decreased albumin as well as CYP3A4 gene expression, while ZEA increased the expression of the HNF4 α and C/EBP α as well as albumin and aldolase genes. For the mycotoxin mixture, albumin, transferrin and aldolase B gene expression was up-regulated while CYP4F3 B gene expression was decreased. Moreover, gene expression profiles appeared extremely opposite at 28 days between AED and TDI doses.

At the highest tested doses, namely the ML doses, as DON and DON

Fig 1. Effect of DON and ZEA alone and in combination at (A) the AED, (B) the TDI and (C) the ML doses on HepaRG cell viability after 14, 28 and 42 days of exposure (mean percentage \pm SEM of cell viability quantified using MTS bioassay, N = 3) * Mean measured cell viability significantly different from negative control (100%) (P < 0.05).



Table 2

	P<0.01 **	P<0.05 *	P>0.05	P<0.05 *	P<0.01 **	Level of gene e	expression (com	pared to control)
	Negative regula	ation		Positive regulation				
	HepaRG after 14 days of exposure			HepaRG after 28 days of exposure				
Gene	Control	DONAED	ZEAAED	DON+ZEAAED	Control	DONAED	ZEAAED	DON+ZEAAED
CYP4F3B	1 ± 0.01	1.06 ± 0.05	0.90 ± 0.04	0.93 ± 0.03	1 ± 0.01	0.94 ± 0.05	0.94 ± 0.06	0.33 ± 0.01 **
CYP3A4	1 ± 0.01	0.97 ± 0.02	$1.67 \pm 0.04 **$	1.15 ± 0.01 **	1 ± 0.01	1 ± 0.09	0.91 ± 0.10	0.1 ± 0.01 **
C/EBPa	1 ± 0.00	1.12 ± 0.04	$1.24\pm0.08*$	1.05 ± 0.02	1 ± 0.03	0.92 ± 0.03	$0.83 \pm 0.03*$	0.47 ± 0.04 **
HNF4a	1 ± 0.03	1.23 ± 0.04**	1.35 ± 0.12*	1.21 ± 0.03**	1 ± 0.02	1.03 ± 0.02	0.96 ± 0.08	0.3 ± 0.01**
Albumin	1 ± 0.01	$0.66 \pm 0.05^{**}$	0.7 ± 0.03**	0.81 ± 0.03**	1 ± 0.01	$0.83 \pm 0.01 **$	0.53 ± 0.01**	0.3 ± 0.02**
Transferrin	1 ± 0.02	1.04 ± 0.01	$1.15 \pm 0.02 **$	1.21 ± 0.05*	1 ± 0.00	0.91 ± 0.02	0.8 ± 0.07	$0.2 \pm 0.02^{**}$
Aldolase B	1 ± 0.02	1.25 ± 0.05**	1.37 ± 0.02**	1.13 ± 0.05	1 ± 0.03	0.96 ± 0.05	1.01 ± 0.03	0.02 ± 0.01 **
	Control	DONTDI	ZEATDI	DON+ZEA _{TDI}	Control	DONTDI	ZEATDI	DON+ZEA _{TDI}
CYP4F3B	1 ± 0.01	1.01 ± 0.01	0.90 ± 0.02 **	0.97 ± 0.01	1 ± 0.01	1.02 ± 0.01	1.03 ± 0.04	$0.88 \pm 0.03 **$
CYP3A4	1 ± 0.01	1.11 ± 0.01 **	1.36 ± 0.01 **	1.05 ± 0.02	1 ± 0.01	$0.92 \pm 0.01*$	1.02 ± 0.01	0.94 ± 0.07
C/EBPa	1 ± 0.00	0.95 ± 0.02	$1.29 \pm 0.09*$	0.97 ± 0.01	1 ± 0.03	1.04 ± 0.01	1.16 ± 0.03*	1.00 ± 0.03
HNF4a	1 ± 0.03	0.93 ± 0.01	1.15 ± 0.08	1.02 ± 0.11	1 ± 0.02	1.13 ± 0.03*	1.22 ± 0.02 **	1.10 ± 0.07
Albumin	1 ± 0.01	$0.79 \pm 0.05*$	$0.6 \pm 0.01*$	$0.38 \pm 0.01 **$	1 ± 0.01	$0.79 \pm 0.04 **$	$1.10 \pm 0.02*$	2.43 ± 0.31**
Transferrin	1 ± 0.02	0.94 ± 0.03	1.07 ± 0.06	$0.92 \pm 0.01*$	1 ± 0.00	1.07 ± 0.12	1.10 ± 0.09	1.16 ± 0.02**
Aldolase B	1 ± 0.02	0.98 ± 0.08	$1.09 \pm 0.01 **$	$1.19 \pm 0.02 **$	1 ± 0.03	$1.14 \pm 0.02*$	1.35 ± 0.13*	1.21 ± 0.02**
	Control	DON _{ML}	ZEA _{ML}	DON+ZEA _{ML}	Control	DON _{ML}	ZEA _{ML}	DON+ZEA _{ML}
CYP4F3B	1 ± 0.01	ND	0.91 ± 0.05	ND	1 ± 0.01	ND	0.98 ± 0.03	ND
CYP3A4	1 ± 0.01	ND	0.91 ± 0.02	ND	1 ± 0.01	ND	1.09 ± 0.03	ND
C/EBPa	1 ± 0.00	ND	0.69 ± 0.03**	ND	1 ± 0.03	ND	$0.84 \pm 0.04*$	ND
HNF4a	1 ± 0.03	ND	$0.49 \pm 0.01 **$	ND	1 ± 0.02	ND	$0.86 \pm 0.01 **$	ND
Albumin	1 ± 0.01	ND	0.37 ± 0.04**	ND	1 ± 0.01	ND	0.84 ± 0.01**	ND
Transferrin	1 ± 0.02	ND	0.58 ± 0.01**	ND	1 ± 0.00	ND	$0.79 \pm 0.04*$	ND
Aldolase B	1 ± 0.02	ND	0.93 ± 0.07	ND	1 ± 0.03	ND	0.93 ± 0.03	ND

Effect of DON and ZEA alone and in combination on the expression of 7 genes of interest in HepaRG cells after 14 and 28 days of exposure (mean \pm SEM fold of control of gene expression quantified using q-PCR, N = 3). Expression was arbitrarily set to 1 in controls for all genes measured; N.D = not detectable.

+ ZEA mixture reduced the cell viability by more than 90% after 14 days of exposure, and 100% after 28 days, no sufficient amounts of RNA, and hence cDNA, were obtained to amplify the different genes. As for ZEA, results showed that, after 14 and 28 days of exposure, expression of C/EPB α and HNF4 α as well as albumin and transferrin genes was down-regulated.

Furthermore, albumin, CYP3A4 and claudin-1 protein expression was assessed by Western blot for each treatment. After 14 days of exposure (Fig. 2A), no significant difference with control was observed for all conditions regarding albumin expression, while CYP3A4 expression was increased after ZEA single exposure at the AED and ML doses. A correlation between the transcription and translation for CYP3A4 was observed at AED doses but not at ML doses. For the tight-junction protein claudin-1, a significant reduction of its expression after DON and ZEA single and combined exposures at the TDI doses was observed, as well as ZEA at the ML dose. After 28 days of exposure (Fig. 2B), albumin expression was significantly decreased after DON + ZEA combined exposure at the AED and TDI doses. Concerning CYP3A4 expression, DON and ZEA at all tested doses seemed to increase its expression, whereas the mixture decreased it. Regarding claudin-1, at the TDI doses, mycotoxin single and combined-exposures decreased its expression, as after 14 days of exposure. In addition, the mixture reduced claudin-1 expression at the AED dose too. Noteworthy, the decrease in albumin and CYP3A4 protein expression at AED dose was correlated with the mRNA expression.

4. Discussion

During the recent years, improvement of analytical methods from single to multi-target methods has allowed highlighting mycotoxin cocontamination of food commodities. In this context, humans and animals can be exposed to several mycotoxins simultaneously through food consumption and it is therefore of great importance to assess the combined toxicity of mycotoxins. Indeed, a mycotoxin cocktail can lead to a possible higher risk of adverse effects than the intake of a single mycotoxin since, among the possible interactions, additive or synergistic effects can be observed (Smith et al., 2016). Noteworthy, for regulation purposes, mycotoxin toxicity is evaluated individually.

Several studies showed the hepatotoxicity of TCT, especially of DON, as well as ZEA, both in vivo and in vitro (Ayed-Boussema et al., 2008; Bradlaw et al., 1985; Cetin and Bullerman, 2005; Knasmüller et al., 1997; Königs et al., 2008; Lafarge-Frayssinet et al., 1981; Mikami et al., 2004), but very few of them looked at their in vitro combined effects on human cells and that in chronic exposure conditions. Indeed, most of them were performed in acute conditions, while the reality of mycotoxin exposure rather corresponds to low doses for long periods (up to a lifetime) in connection with the consumption of mycotoxincontaminated food. Moreover, animal models are frequently used to identify hepatotoxic drugs whereas more than 50% of drugs that induce liver injury in human clinical trials are not hepatotoxic to animals (Olson et al., 2000). Therefore, human liver cells are more accurate for in vitro evaluation of drug toxicity. In particular, HepaRG cells provide a suitable alternative to primary human hepatocytes for chronic hepatotoxicity studies. The present study was designed to evaluate, in chronic exposure conditions, the individual and combined effects of DON and ZEA, one most frequently occurring mycotoxin combination, both at the cellular (cytotoxicity) and molecular (regulation of hepatic key factors) levels.

Cell viability results showed that DON and ZEA, alone and in combination, at both AED and TDI doses, did not induce significant cell death even after 42 days of exposure as measured by the MTS assay. However, cell viability results also revealed that DON at the ML dose induced a high cytotoxicity on HepaRG cells from 14 days of exposure. Interestingly, in acute exposure conditions (48 h), at similar doses and using the same MTS assay, no significant cytotoxicity was observed on HepaRG cells (data not shown), highlighting the relevance of chronic



Fig. 2. Effect of DON and ZEA alone and in combination at the AED, TDI and ML doses on albumin, claudin-1 and CYP3A4 expression after 14 (A) and 28 days (B) of exposure (mean percentage \pm SEM of protein expression, N = 3) * Mean measured protein expression significantly different from negative control (arbitrarily set to 1) (P < 0.05).

exposure experiments to assess cell viability. Concerning ZEA single exposure, no significant cell mortality was observed at the ML doses for all tested exposure periods, as for AED and TDI doses. This is not surprising as ZEA is known to be usually non-lethal and has been reported to be less toxic than DON on hepatocyte models (Wan et al., 2013; Wentzel et al., 2016). Finally, regarding the mycotoxin mixture at the ML doses, the same toxicological effect as the one determined for DON alone was observed thus indicating no antagonistic or synergistic effect. As ZEA alone did not significantly modify the cell viability, the mixture effect can be considered to be additive. Nevertheless, since mycotoxin combined effects on cell viability are time-, dose- and mixture-dependent, these findings should not be considered as representative of all fusariotoxin mixtures present in foodstuffs, especially as synergism could potentially occurred.

To obtain a better view of the potential chronic impact of mycotoxins mixtures on HepaRG functional state and metabolic pathways, expression of relevant hepatocyte-specific factor associated genes was measured in HepaRG cells exposed for 14 and 28 days to DON and/or ZEA. Contrary to other human liver cell lines, HepaRG cells maintain many liver-specific functions with levels of expression close to those found in primary human hepatocytes, including expression of major cytochromes P450, membrane transporters, nuclear receptors and Phase II enzymes (Andersson et al., 2012; Aninat et al., 2006; Anthérieu et al., 2010, 2012; Gerets et al., 2012; Jossé et al., 2008). The selected genes encoded the following liver-specific factors: the cytochrome P450 CYP3A4 and CYP4F3 B enzymes (involved in phase I detoxification metabolism), the nuclear C/EBPa and HNF4a transcription factors, the transferrin and albumin plasma proteins (liver-specific proteins) and the aldolase B glycolytic enzyme. More specifically, CYP3A4 is the most important and abundant P450 expressed in human liver, contributing to

the catabolism of approximately 50% of the drugs in use today (Guengerich, 1999). On the other hand, the CYP4F3 B isoform is the key enzyme for ω -hydroxylation of arachidonic acid in liver cells and thus may exert important functions in lipid homeostasis and in inflammatory diseases (Antoun et al., 2008). Furthermore, most of P450 expression are under strict control of various transcription factors that are regulated during differentiation (Burk and Wojnowski, 2004). For instance, the hepatocyte nuclear factor 4α (HNF4 α) and the liver-enriched nuclear factor CCAAT/enhancer-binding protein (C/EBP) a are key regulators controlling the expression of several hepatic metabolic processes and thus are potentially entailed in the regulation of these P450 enzymes. Madec et al. (2011) reported that the HepaRG cell differentiation process was characterized by a gradual expression increase in HNF4a, CYP3A4, CYP4A11 and CYP4F3 B as well as specific enzymes of the glucose metabolism such as aldolase B, highlighting that various P450 might share common mechanisms controlling gene expression mediated by liver-specific transcription factors and nuclear receptors. Furthermore, a reduction of the expression of the abovementioned genes might highlight a loss of specialized liver functions or could even suggest a dedifferentiation of the hepatocytes (Godoy et al., 2009). Here, even if only 0.4% of DMSO was used in the cell medium (while 2% is recommended to potentiate the differentiation process), we assumed the cells were differentiated after several weeks to 100% confluence. To verify this hypothesis, we compared the expression levels of CYP3A4 and CYP4F3 B genes in undifferentiated cells (only one week after seeding) and in cell from controls from the present study (after 14 and 28 days exposure). Results showed a significant higher expression for both genes in cells exposed several days, confirming the differentiated state of the HepaRG cells (data not shown). The obtained results presented in Table 2 showed, at the lowest tested doses (namely

AED) after 14 days of exposure, that several liver-specific genes seemed to be regulated following DON and ZEA single and co-exposures, and that even if no significant effect on cell viability reduction was observed. Different results were observed depending on the considered mycotoxin or mixture, with some similarities among the different conditions (e.g. reduction of albumin gene expression). Moreover, ZEA seemed to induce more changes at the gene level than DON or even the mixture. In addition, all genes affected by the mixture were common with ZEA single-exposure. Interestingly, after 28 days of DON and ZEA single exposures, effects on the liver-specific gene expression seemed to be lower than after 14 days of exposure suggesting a possible cell adaptation towards these two toxins at low cytotoxic doses. Indeed, only albumin gene expression was highly down-regulated with these fusariotoxins. It was found that the reduction in serum albumin concentration is proportional to the reduction in liver synthesis rates (Levitt and Levitt, 2016). Thus, the observed decrease in albumin expression is a serious sign of cellular function disturbance. It's not the case for the mixture for which a decrease in gene expression was observed at 28 days of exposure for all the targets. These findings highlighted a major change of the metabolic activity of HepaRG cells. The combined significant reduction of albumin and CYP3A4 mRNA and protein expression at 28 days of DON-ZEA exposure is of particular interest. The increase in CYP3A4 protein expression after 14 and 28 days of ZEA single exposure as well as 28 days of DON exposure highlighted a CYP3A4-dependent catalytic activity induced by these treatments. Several authors have been interested in mycotoxin-induced hepatotoxicity and they reported, for instance, that hepatotoxicity of aflatoxin B1 was CYP3A4-dependent too (Gallagher et al., 1996; Sivertsson et al., 2010; Ueng et al., 1995). More particularly, toxicity of aflatoxin B1 is mediated by the formation of the toxic metabolite 8,9epoxide, formed by CYP1A2 and 3A4, that explain that differentiated HepaRG cells are more sensitive to this toxin compared to undifferentiated cells which poorly expressed major cytochromes P450 (Guillouzo et al., 2007).

Regarding the TDI doses, as for the AED doses, ZEA induced the most important effect on gene expression levels, followed by DON + ZEA combination and DON single exposure. These observations are in accordance with the study of Königs et al. (2008) which concluded that DON is neither metabolized by human primary hepatocytes and HepG2 cell line. However, contrary to the observed effects at the AED doses, after 28 days of exposure, effects on gene expression were not limited compared to 14 days for DON and ZEA individual exposures. Interestingly, the mixture significantly up-regulated albumin, transferrin and aldolase B gene expression, highlighting this time a high metabolic activity of HepaRG cells and suggesting that the potential cellular adaptation observed at 28 days at AED doses would not be observed at TDI doses. Furthermore, claudin-1 protein expression was significantly reduced for all treatments after 14 and 28 days of exposure, suggesting an increase in the cell monolayer permeability through tight junctions. Other studies have reported that DON and ZEA affected the barrier function of intestinal epithelial cells through the decrease in expression of claudin proteins (Marin et al., 2015; Pinton et al., 2010).

As for the ML doses, ZEA alone appeared the less toxic among all treatments since DON and DON + ZEA induced about 100% of cell mortality at these doses (this effect of the mixture being very highly correlated to the DON individual effect). After 14 days, ZEA mono-exposure seemed to induce an expression reduction of most of the tested genes. In addition, similar effects were observed after 14 and 28 days for this toxin, even if the reduction of gene expression level was less significant after 28 days. These results highlighted the loss of HepaRG cell functionality in the tested conditions. In addition, after 14 days, claudin-1 expression of was down-regulated at the protein level whereas no significant difference with the control could be detected after 28 days.

Thus, these results highlighted that effects on gene expression levels

were time-, dose- and toxin-dependent. In addition, even if after 14 days exposure the mixture shared similar mechanisms with DON or ZEA alone, after 28 days the mixture showed different effects on gene expression compared to the toxins alone. These findings emphasized that different cellular pathways responded to the different single and combined mycotoxins.

Concerning the regulation aspects, it is worth noting that the DON and ZEA TDI values have been established by the JECFA and SCF based on the data from a 2-year feeding study conducted on mice (Iverson et al., 1995) and a 15-day period on sows (Edwards et al., 1987b, 1987a), respectively. A safety factor of 100 was applied to the no-effect doses to obtain the TDI values which are therefore 1 µg/kg bw/d for DON (EFSA, 2007: JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2001; SCF, 2000) and 0.2 µg/kg bw/d for ZEA (SCF, 2000). Then, ML values set by the European Commission have been established taking into account risk assessment data as well as TDI values (EC, 2006). As animal models differ from humans, ML may underestimate the impact on humans as exampled by the high DON cytotoxic effects observed in this study. However, in this study, the tested mycotoxins were directly spiked into the culture medium whereas, in vivo, only a small amount of mycotoxins present in cereals is absorbed in the human digestive tract, meaning that the final mycotoxin exposure doses in the liver are lower. Noteworthy, the absorbed amount may vary according to a given mycotoxin. For example, Warth et al. (2013) observed that DON was rapidly excreted after ingestion with a daily excretion rate of 68% on average in human urine samples, which is close to the one reported by Turner et al. (2010) (i.e. 72%). On the contrary, ZEA is generally rapidly absorbed after intake. Warth et al. (2013) observed an average daily excretion rate of 9.4% in human urine and similarly, approximately 10-20% were recovered in 24 h urine samples by Mirocha et al. (1981). Nevertheless, in humans, toxicokinetic studies regarding these mycotoxins are still scarce. On the other hand, while the actual mycotoxin exposure doses may have been overestimated, the repeated exposure to DON and ZEA was only investigated over a few weeks while humans are certainly exposed throughout their lifetime to mycotoxin mixtures. In this context, it could be of interest to compile data from in vivo studies with those obtained in vitro on human cells to better establish ML values.

5. Conclusion

In the present study, cytotoxicity assessments revealed a high cytotoxicity of DON alone and in combination with ZEA in chronic exposure at the maximum level permitted in cereals intended for direct human consumption by the European regulation. Furthermore, at lower doses, after 28 days of exposure, the mixture induced major changes of the metabolic activity of HepaRG cells, even if no significant effect on cell viability was observed in comparison to control condition. Thus, although these results have been obtained *in vitro* and therefore did not take into account the inherent interactions with the whole organism as well as the complexity of food ingestion (i.e. interactions between foods during meal consumption), they raised the question of the toxicological impact of a long-term chronic exposure to mycotoxins (alone and in combination). Overall, this study highlights the need to better take into account both chronic exposure and multi-mycotoxin contamination in toxicological studies and that potentially at the regulation level.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Les points marquants de cette étude sont les suivants :

- La teneur maximale autorisée par la réglementation européenne (TM) dans les céréales destinées à l'alimentation humaine pour le DON, seul et en présence de la ZEA, induit une très forte mortalité cellulaire dès 14 jours d'exposition dans les conditions *in vitro* testées.
- Des effets cytotoxiques similaires au DON seul sont observés pour le mélange, quelle que soit la durée d'exposition, soulignant un effet combiné additif du DON et de la ZEA sur la réduction de la viabilité cellulaire.
- A des doses plus faibles, d'importantes variations des niveaux d'expression de certains gènes codant pour des fonctions spécifiques des hépatocytes sont observées alors qu'aucun effet significatif sur la viabilité n'a été relevé.
- Après 14 jours d'exposition, l'expression de moins de gènes semble être impactée par le mélange DON+ZEA que par les toxines seules. A l'inverse, après 28 jours, le mélange semble induire des changements majeurs sur l'activité métabolique des cellules, en ciblant des gènes différents de ceux affectés par le DON et la ZEA présents individuellement.
- Bien que les résultats aient été obtenus *in vitro*, et ne tiennent donc pas compte de la complexité des interactions des mycotoxines avec les aliments et l'organisme, ceux-ci suggèrent que certaines valeurs réglementaires pourraient sous-estimer l'impact toxicologique en condition chronique voire dans le cas de multi-contaminations.

La figure 12 schématise de façon synthétique les principaux résultats obtenus dans cette étude.



Figure 12 : Représentation schématique des principaux effets biologiques observés dans l'étude n°4.

Partie IV : Utilisation de systèmes de coculture *in vitro* pour l'évaluation de la cytotoxicité aiguë des fusariotoxines seules et en mélange sur les lignées humaines Caco-2, THP-1 et HepaRG

Etude n°5:

In vitro co-culture models to evaluate acute toxicity of individual and combined mycotoxin exposures on Caco-2, THP-1 and HepaRG human cell lines

Toxicology and Applied Pharmacology (https://doi.org/10.1016/j.cbi.2017.12.004)

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Le dernier volet de cette thèse est consacré à l'étude des effets individuels et combinés du DON et de la ZEA sur différents modèles cellulaires humains superposés *via* des systèmes de coculture nécessitant l'utilisation d'inserts, aussi appelés « transpuits ». En effet, *in vivo*, les cellules travaillent en relation étroite avec d'autres cellules et tissus, tel un réseau, et peuvent être affectées séquentiellement par l'exposition aux mycotoxines (par exemple, cellules de l'intestin, puis cellules sanguines, puis hépatocytes). Les systèmes de coculture dans des conditions *in vitro* ou *ex vivo* peuvent donc représenter un pas en avant pour s'approcher, voire mimer, la situation *in vivo*. Ceci représente un challenge d'autant plus important dans un contexte visant à réduire les tests sur animaux. Quelques auteurs ont d'ailleurs déjà mis en place de tels systèmes de culture comprenant des macrophages THP-1 avec des adipocytes, des cellules intestinales, des cellules dendritiques ou des lymphocytes T (Chanput 2014).

Ici, nous avons choisi d'étudier les effets toxicologiques induits après 48h d'exposition au DON, à la ZEA et au mélange DON+ZEA sur (i) les hépatoblastes HepaRG et (ii) les monocytes THP-1 après passage de la barrière intestinale mimée par les cellules Caco-2 cultivées dans les inserts, afin d'évaluer dans quelle mesure l'épithélium intestinal protège les cellules hépatiques et les monocytes de l'exposition aux mycotoxines ; ainsi que sur (iii) les monocytes THP-1 mais, cette fois, après passage à travers la barrière formée par les cellules

hépatiques HepaRG cultivées dans les transpuits. Une quatrième coculture dite « en contact », faisant intervenir les 3 modèles cellulaires, a également été réalisée. Dans cette dernière condition, les cellules Caco-2 ont été ensemencées au niveau de la face supérieure des inserts (comme pour les cocultures (i) et (ii)), alors que les HepaRG ont été ensemencées sur la face inférieure de ces mêmes inserts. Dans un tel système, seule la membrane poreuse sépare les Caco-2 des HepaRG. Enfin, les THP-1 ont été ensemencées dans le compartiment inférieur du système (comme lors des cocultures (ii) et (iii)). Afin de pouvoir comparer les résultats obtenus en co-culture avec ceux des mono-cultures, toutes les cellules ont été utilisées dans un état indifférencié, c'est-à-dire à l'état d'entorocytes immatures pour les Caco-2, à l'état de moncoytes pour les THP-1 et en tant qu'hépatoblastes pour les HepaRG.

Après 48h d'exposition, la viabilité des cellules cultivées dans le compartiment inférieur (c'est-à-dire les HepaRG pour la coculture (i) et les THP-1 pour les cocultures (ii) et (iii)) a pu être mesurée *via* un test MTS. La perméabilité membranaire des cellules Caco-2 cultivées dans les inserts (dans le cas des cocultures (i) et (ii)) a été évaluée *via* un test TEER. Enfin, afin d'estimer la quantité de mycotoxines traversant cette barrière, les concentrations en DON et ZEA présentes dans le milieu ont été dosées par LC-MS dans les surnageants de culture des compartiments inférieurs (ou abluminaux).

La figure 13 schématise de façon synthétique les principaux effets biologiques évalués dans cette étude.



Figure 13 : Résumé schématique des différents effets biologiques et conditions évalués dans l'étude n°5.

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In vitro co-culture models to evaluate acute cytotoxicity of individual and combined mycotoxin exposures on Caco-2, THP-1 and HepaRG human cell lines



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ABSTRACT

Deoxynivalenol (DON) and zearalenone (ZEA) are mycotoxins primarily produced by Fusarium species and commonly co-occur in European grains. Some in vitro studies reported synergistic combined effects on cell viability reduction for these two natural food contaminants. However, most of these studies were carried out on conventional cell culture systems involving only one cell type and thus did not include cell-cell communication that is closer to in vivo conditions. In this context, we developed easy bi- and tri-culture systems using the Caco-2 (intestinal epithelial cells), THP-1 (monocytes) and HepaRG (hepatic cells) human cell lines in a proliferating state. Individual and combined cytotoxic effects of DON and ZEA were then assessed using co-cultures during 48 h. In bi-culture systems, results showed that only the highest tested dose of ZEA (IC₃₀) induced a significant reduction in THP-1 viability with both Caco-2 and HepaRG cells cultured in transwells above. On the contrary, only the highest tested dose of DON (IC₃₀) significantly affected HepaRG cell viability located under the Caco-2 cell monolayer. In addition, the DON + ZEA combination seemed to induce higher cytotoxicity than each toxin alone. Mycotoxin quantification in the abluminal compartment by Q-TOF LC-MS suggested uptake of both mycotoxins by the different cell lines. According to the co-culturing cell type, possible cell-cell interactions were also observed. Finally, in the tri-culture system, no cytotoxic effects were observed, regardless of the treatment. These findings highlighted the importance of the proposed models to better decipher toxicological impacts of mycotoxins on more complex cellular systems.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi, ubiquitous in nature and frequently occur in food and feed worldwide. As fungi are able to simultaneously and/or successively produce several mycotoxins, and as a product may be contaminated by several mycotoxigenic fungi, mycotoxin multi-contamination is of particular concern. This is especially true as numerous studies have highlighted their potential additive and even synergistic toxic effects both *in vitro* and *in vivo* [11,33]. Among the infinite number of potentially encountered mycotoxin mixtures in commodities, the co-occurrence of deoxynivalenol (DON)/zearalenone (ZEA) is one of the most widespread in European grains [33]. Both mycotoxins are produced by some *Fusarium* species, such as *F. culmorum*, *F. crookwellense* and *F. graminearum*, which

may explain their frequent co-occurrence in grains [33]. Fusariotoxins have been described to have hepatotoxic, hematotoxic, genotoxic and immunotoxic effects. More specifically, DON significantly affects the immune system resulting in acute toxicity characterized by vomiting, nausea and diarrhea in humans and many experimental animal models. At the molecular level, DON disturbs normal cell function by inhibiting protein synthesis. Other mechanisms have been proposed to explain its toxicity, including membrane function, intercellular communication and calcium homeostasis impairments [23,24]. DON is also known to activate protein degradation by the proteasome [49]. Regarding ZEA, also classified as mycotoestrogen, it induces estrogen gene activation due to its ability to bind estrogen receptors in target cells [38,47]. ZEA causes functional and morphological alterations on reproductive organs in farm animals and occasionally hypoestrogenic syndromes in humans

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Abbreviations: DMSO, Dimethylsulfoxide; DON, Deoxynivalenol; IC₁₀, Inhibitory concentration 10%; IC₃₀, Inhibitory concentration 30%; LC-MS, Liquid chromatography coupled to mass spectrometry; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); Q-TOF, Quadrupole time-of-flight; TCT, Trichothecenes; TEER, Transepithelial/transendothelial electrical resistance; ZEA, Zearalenone

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[48]. In addition, it is suspected to be implicated in precocious puberty development in girls [18]. Nevertheless, the exact mechanism of ZEA toxicity has not been completely unraveled [48].

As mycotoxins are food contaminants, they firstly interact with the gastrointestinal epithelium upon ingestion. Then, mycotoxins may consequently induce local toxicity and/or cross this barrier to reach the bloodstream. DON is the most prominent example of a mycotoxin primarily associated with intestinal integrity impairment, as reported by several authors using the human intestinal epithelial Caco-2 cells [50–53]. In addition, DON has been shown to affect nutrient uptake and to induce inflammatory response in Caco-2 cells [50,54-56] [25]. The mucosal surface of the gastrointestinal tract is lined by a monolaver of intestinal epithelial cells joined together at their apical poles by tight junctions, and forming a bulkhead that separates the luminal contents from the immune cells underneath. Thus, intestinal cell models are commonly used to study absorption, metabolism and bioavailability of drugs and xenobiotics. More specifically, the human Caco-2 cancer cell line has been reported to be a suitable model to study mycotoxin toxic effects on intestinal barrier functionality using specific endpoints, such as trans-epithelial electrical resistance to measure paracellular permeability and/or expression of tight junction proteins [2,5]. On the other hand, the THP-1 human leukemia monocytic cell line has been extensively used to study monocyte/macrophage functions, mechanisms, signaling pathways as well as nutrient and drug transport. This cell lineage expresses characteristic markers of monocytes in suspension, and is known to be a suitable and reliable model to study monocytes [4]. Furthermore, as the liver is our main detoxification organ, it also represents a main target of mycotoxins and, therefore, human hepatic cells constitute one of the most relevant in vitro standard models for xenobiotic metabolism and toxicity studies. The HepaRG cell line, derived from a human hepatocellular carcinoma, is of particular interest as it possesses both the primary hepatocyte metabolic performances and the hepatic cell line growth capacity [12,22]. More specifically, and contrary to other human liver cell lines, HepaRG cells maintain many liver-specific functions including expression of major cytochromes P450s, membrane transporters, nuclear receptors and Phase II enzymes [1,12].

To this date, *in vitro* studies are mainly performed on individually targeted cell lines. However, *in vivo*, cells always work in cross talk with other cells and tissues as a network. Thus, co-culture systems in *in vitro* conditions may represent a step forward to mimic *in vivo* conditions. In addition, cultured cells may offer a suitable alternative to *in vivo* animal testing and represent an indispensable tool to elucidate mycotoxin action mechanisms. In this context, the aim of this work was to develop simple *in vitro* co-culture models to investigate mycotoxin cytotoxicity on different human cell lines. Then, these models were applied to evaluate individual and combined DON and ZEA toxicological effects.

2. Materials and methods

2.1. Mycotoxins

Mycotoxin standards, namely DON (MW = 296.35 g/mol; CAS#51481-10-8) with purity > 98% and ZEA (MW = 318.36 g/mol; CAS#17924-92-4) with purity > 99%, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) to final concentrations of 10^{-3} M for DON and 10^{-2} M for ZEA, and stored at -20 °C.

2.2. Cell culture conditions

2.2.1. Caco-2 culture

Caucasian colon adenocarcinoma cells (Caco-2) were obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK). They were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% heatinactivated fetal bovine serum (FBS, Biowest, Nuaillé, France), 2 mM of L-glutamine (Sigma-Aldrich), 1% penicillin 10 000 Units/ml and 1% streptomycin 10 000 μ g/ml (Biochrom GmbH, Berlin, Germany) (changed every 2–3 days) and incubated at 37 °C with 100% relative humidity (RH) under 5% CO₂ until near confluence. Then, Caco-2 cells were harvested with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Biowest), and reseeded in a fresh medium as a function of cell density, as recommended by ECACC.

2.2.2. HepaRG culture

Human hepatic cells (HepaRG) were acquired from Biopredic International (Saint Grégoire, France). They were cultured in William's E medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin 10 000 Units/ml and 1% streptomycin 10 000 µg/ml, 5 µg/ml insulin (SAFC, St. Louis, MO USA) and 50 µM hydrocortisone hemisuccinate (Sigma-Aldrich) at 37 °C with 100% RH in a 5% CO₂ atmosphere. Culture medium was replaced every 2–3 days with fresh growth medium. The cells were passaged every 2 weeks at a density of 2.7×10^4 cells/cm² by a short time exposure (< 5 min) with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA, and reseeded in a fresh medium.

2.2.3. THP-1 culture

Human monocytic leukemia cells (THP-1) were acquired from the European Collection of Cell Cultures (ECACC; number 88081201, Salisbury, UK). THP-1 suspensions were grown in RPMI-1640 medium (Biowest) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin 10 000 Units/ml and 1% streptomycin 10 000 µg/ml at 37 °C with 100% RH in a 5% CO₂ atmosphere. Cells were grown to a density between 0.2 and 1 \times 10⁶ cells/ml as recommended by ECACC. Culture medium was replaced every 2–3 days with fresh growth medium.

2.3. Co-culture model construction

Four co-culture systems were constructed (Fig. 1): A, non-contact biculture involving Caco-2 and HepaRG; B, non-contact bi-culture involving Caco-2 and THP-1; C, non-contact bi-culture involving HepaRG and THP-1 and D, tri-culture involving Caco-2 in contact with HepaRG and THP-1.

2.3.1. Bi-culture construction

Three different *in vitro* bi-culture models were constructed using the three different human cell lines, *i.e.* Caco-2, HepaRG and THP-1 (Fig. 1). Bi-cultures A and B were non-contact co-culture models in which the culture inserts contained the Caco-2 cells which were cultured together with either HepaRG cells or THP-1 cells respectively, located on the bottom of the 24-well culture plate. Bi-culture C corresponded to HepaRG in the hanging cell culture inserts and THP-1 located on the bottom of the culture plate.

For construction of these non-contact bi-cultures, Caco-2 or HepaRG cells were seeded into 24-well cell culture inserts on a semipermeable support membrane (cell culture insert 0.4 µm pore size; Falcon, Corning, New-York, USA) at a density of 1.7×10^5 cells/cm² (*i.e.* 5×10^4 cells/insert, corresponding to a confluent cell monolayer) and were incubated for 24 h under normal culture conditions with the appropriate culture medium. Then, the cell culture inserts containing Caco-2 or HepaRG cells were placed into the 24-well culture plates (Falcon) containing THP-1 cells seeded at a density of 3×10^5 cells/ml (i.e. 4×10^5 cells/well) or HepaRG cells seeded 24 h before at a density of 1.7 \times $10^5\,cells/cm^2$ (i.e. 2.7 \times $10^5\,cells/well).$ Both inserts and wells were supplied with media and incubated 48 h after mycotoxins addition in the cell-culture inserts. The final volume of the medium was 700 μ l in the bottom of each well (named abluminal compartment) and 200 µl in the hanging inserts (named luminal compartment). All bi-culture systems using HepaRG cells were maintained in HepaRG medium and only bi-culture B was nurtured with Caco-2 medium.



Fig. 1. Schematic representation of the four constructed *in vitro* co-culture systems: A) non-contact Caco-2 and HepaRG bi-culture; B) non-contact Caco-2 and THP-1 bi-culture; C) non-contact HepaRG and THP-1 bi-culture and D) Caco-2, HepaRG and THP-1 tri-culture.

2.3.2. Tri-culture construction

In the tri-culture model, Caco-2 cells were cultured on the upper side of the culture inserts, while HepaRG hepatocytes were cultured on the bottom side of the culture inserts. THP-1 cells were seeded on the bottom of the culture plates.

When constructing the tri-culture, the hanging cell culture insert was first turned upside down in 6-well culture plates. Before seeding the HepaRG cells, another insert without support membrane was placed against the first insert to avoid any leakage of cells and medium. About 5×10^4 HepaRG cells were resuspended in 100 µl of medium and then seeded on the bottom face of the overturned insert. The system was maintained for 3–4 h until cell adhesion. The inserts carrying the HepaRG cells on their bottom face were then placed hanging into a 24-well culture dish containing THP-1 cells (4×10^5 cells/well). Then, Caco-2 cells were seeded on the upper side of the culture inserts (5×10^4 cells/insert). As for bi-culture models, both inserts and wells were supplied with 200 µl and 700 µl of HepaRG cell culture medium, respectively, and incubated 48 h after mycotoxin addition in the cell-culture inserts. For all co-cultures, cell lines were in a proliferating state.

2.4. Mycotoxin exposure

2.4.1. Cytotoxicity evaluation via MTS assay

The individual and combined cytotoxic effects of DON and ZEA were explored after 48 h exposure on the cells located on the bottom of the 24-well culture plates (*i.e.* HepaRG for bi-culture A and THP-1 for co-cultures B, C and D). For each toxin, two concentrations were tested, corresponding to the IC_{10} and IC_{30} values (concentrations inhibiting 10% and 30% of cell viability, respectively) previously obtained on Caco-2 (Figure S1) and HepaRG [35] mono-culture experiments after 48 h exposure with these mycotoxins (Table 1). More particularly, IC values obtained on Caco-2 were used for co-cultures A, B and D, while those obtained on HepaRG were employed for co-culture C. These inhibitory concentrations were calculated from theoretical dose-response curves established with Microsoft TableCurve 2D Software v.5.01 (Microsoft, Redmond, WA, USA) and obtained by plotting the percentage of cell viability against the log of mycotoxin concentrations. The final

concentration of solvent (DMSO) in cell culture containing mycotoxins did not exceed 0.5%. Appropriate control cultures without mycotoxin but containing the same amount of solvent were included as negative controls. No significant difference was observed between the solvent control (with DMSO only) and cells without DMSO (data not shown).

Mycotoxin cytotoxicity was evaluated using the Promega CellTiter 96AQ_{ueous} Non-Radioactive cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instructions and as previously described by Smith et al. [34]. Cell viability obtained for the negative control was defined as 100%. Cell viability mean percentages of three independent experiments \pm standard error of the mean (SEM) were used for statistical analyses.

To evaluate the mycotoxin combined effects, two conceptual models were applied: the "linear interaction effect" [32], also called "response additivity", and the "Bliss independence model" [3], also named "independent joint action", which have been recently reviewed by Foucquier and Guedj [9]. A combination index (CI) was calculated for each model. This index is recognized as a standard measure of combination effect and indicates a higher (CI < 0.9), lower (CI > 1.1) or similar ($0.9 \leq CI \leq 1.1$) effect than the expected additive effect. The CI of the "linear interaction effect" model can be calculated as follows:

CI = [observed effect(mycotoxin1) + observed effect(mycotoxin2)]

÷ observed effect (mycotoxin1 + mycotoxin2)

The CI of the "Bliss independence criterion" can be calculated as follows:

CI = [observed effect (mycotoxin1) + observed effect (mycotoxin2)

- observed effect (mycotoxin1) \times observed effect (mycotoxin2)]
- ÷ observed effect (mycotoxin1 + mycotoxin2)

2.4.2. Barrier integrity assessment using TEER measurement

The cell monolayer integrity of Caco-2 cells exposed for 48 h to DON, ZEA and DON + ZEA mixture, as well as the control, in bi-cultures A and B, was controlled by the transepithelial electrical resistance (TEER) measurement. TEER was measured under sterile conditions

Table 1

Tested mycotoxin concentrations in the different co-culture models. All concentrations correspond to the mycotoxin doses added in the luminal compartment.

Co-culture	А	В	С	D
IC10 DON	1.6 μΜ	1.6 μM	0.2 μΜ	1.6 μM
IC30 DON	3 μM	3 μM	2.3 μM	3 μΜ
IC10 ZEA	24 µM	24 µM	20 µM	24 µM
IC ₃₀ ZEA	31 µM	31 µM	33 µM	31 µM
IC ₁₀ DON + ZEA	1.6 μM + 24 μM	1.6 μM + 24 μM	0.2 μM + 20 μM	1.6 µM + 24 µM
IC_{30} DON + ZEA	$3 \ \mu M + 31 \ \mu M$	$3 \ \mu M + 31 \ \mu M$	2.3 μM + 33 μM	$3 \ \mu M + 31 \ \mu M$



Fig. 2. Cell viability assessed on HepaRG or THP-1 cells depending on the co-culture combination after 48 h exposure to DON, ZEA and DON + ZEA at their respective IC_{10} and IC_{30} (mean percentage \pm SEM of cell viability quantified using MTS bioassay, N = 3) * = cell viability measured mean significantly different from the negative control (100%) (P < 0.05).

using an epithelial voltohmmeter, the Millicell ERS system (Millipore Co. Bedford, MA) according to the manufacturer's instructions. TEER values, were recorded and expressed as $\Omega \times \text{cm}^2$ on the basis of the following equation: $TEER = (R - R_b) \times A$, where R is the resistance of filter insert with cells, R_b is the resistance of the filter alone and A is the growth area of the filter in cm². TEER was expressed in % compared to the control Caco-2 cells unexposed to mycotoxins but treated with DMSO. No significant difference was observed between the solvent control and cells without DMSO (data not shown). Three independent experiments were performed for each condition.

2.4.3. Mycotoxin quantification in the abluminal compartment by Q-TOF LC-MS $\,$

In order to evaluate the amount of mycotoxin(s) able to go through the cell monolayer after 48 h exposure to DON, ZEA and DON + ZEA mixtures, culture media from the abluminal compartment of bi-cultures A, B and C was collected, dissolved in acetonitrile (1:1) and filtered through a 0.2 µm PTFE membrane syringe 4 mm filter (GE Healthcare Life Sciences, Little Chalfont, UK) in an amber vial. A LC-MS/ Quadrupole Time-of-Flight (Q-TOF) was used in order to separate and identify the extracted metabolites from each sample. The Agilent 1290 Series HPLC system included a binary pump and degasser, well plate autosampler with thermostat and a thermostated column compartment. Two microliters of each sample were injected in the system and separation was achieved using a ZORBAX Extend-C18 column (2.1 \times 50 mm and 1.8 μ m, maximum pressure 600 bar) (Agilent, France) that was maintained at 35 °C throughout the entire chromatographic run. The flow rate was set to 0.3 ml min⁻¹ using the following mobile phase: solvent A (milli-Q water + 0.1% formic acid + 10 mM ammonium formate) and solvent B (100% acetonitrile). Solvent B was maintained at 5% for the first 4 min, followed by a gradient of 5-100% of solvent B for 16 min, and then maintained at 100% during a 5-min post-time to equilibrate the column to original run conditions. Metabolites were detected using an Agilent 6530 Series Accurate-Mass O-TOF mass spectrometer with electrospray ionization (ESI) source operated in positive and negative ion modes. Mass spectrometer conditions were as follows: capillary voltage, 4 kV; source temperature, 325 °C; nebulizer pressure, 50 psig; drying gas, 12 L min⁻¹ and ion range, 100–1000 m/z. LC-MS/Q-TOF calibrations were performed before each run following the mass spectrometer manufacturer's instructions. Relative DON and ZEA quantification was carried out using the prepared mycotoxin standards. For quantification, an 8 point linear range from 0.01 to 50 µM for both mycotoxins was prepared in acetonitrile and linearity (R2) for each standard curve was determined to be > 0.99. Some points were also prepared in the culture medium diluted in acetonitrile (1:2) and no matrix interference was observed. DON could be quantified using the [M-Na]⁺ 319.115 m/ z ion in ESI + mode, while ZEA quantification was performed using the $[M-H^+]^-$ 317.139 *m/z* ion in ESI- mode. Table S1 provides the Q-TOF LC-MS analyte parameters used for DON and ZEA identification and quantification.

2.5. Statistical analysis

Data were expressed as mean values of three independent experiments \pm SEM. Statistical analyses were performed using the unpaired Student's t-test for comparison with control. Values of P < 0.05 were accepted as significant.

3. Results

3.1. Effects of individual and combined mycotoxin exposures on abluminal cell viability

Results from the cell viability experiments are illustrated in Fig. 2. For all bi-culture models, DON and ZEA IC10 doses, used alone and in mixture, did not significantly reduce abluminal cell viability. Regarding bi-culture A, only DON, alone or mixed with ZEA using IC₃₀ doses, significantly reduced HepaRG cell viability after 48 h exposure. More particularly, DON IC30 induced about 20% cell mortality, and almost 40% when combined with ZEA. On the contrary, for bi-culture B, only ZEA and the DON + ZEA mixture at IC₃₀ values significantly reduced THP-1 cell viability by about 25% and 42%, respectively. When the Caco-2 cell monolayer was replaced by the HepaRG cell monolayer (biculture C), similar results were observed for THP-1 cell viability. More particularly, the IC₃₀ of ZEA reduced THP-1 cell viability by nearly 20%, while in combination with DON, the mixture induced 25% cell mortality. Finally, for the tri-culture D, no significant reduction in THP-1 cell viability was observed with DON and ZEA IC10 and IC30 exposures (obtained in this study for Caco-2 cells).

Mycotoxin interactions characterized by the two conceptual mathematical models revealed a synergistic combined effect of DON with ZEA in A, B and C bi-cultures for the highest tested dose (IC_{30}). For the IC_{10} value, as well as for the tri-culture D, additivity (meaning no interaction) was observed on the cell viability reduction (Table 2).

3.2. Gastrointestinal epithelial cell monolayer integrity evaluation after individual and combined mycotoxin exposures

Results from the TEER measurements (Fig. 3) highlighted significant effects for almost all treatments on the Caco-2 cell monolayer integrity. More specifically, when Caco-2 were cultured with THP-1, cell monolayer integrity seemed to be affected by all treatments while, when the Caco-2 were cultured with HepaRG cells, only ZEA and the mycotoxin mixture appeared to significantly reduce cell monolayer integrity at both tested concentrations.

3.3. Mycotoxin quantification in the abluminal compartment

Measured mycotoxin concentrations in the abluminal compartment of the different co-culture models after 48 h incubation are reported in Table 3. For all co-culture combinations, results showed that similar mycotoxin concentrations were measured in the abluminal compartment in both mono- and co-exposure experiments for a considered mycotoxin. Regarding bi-culture A, mainly DON was detected in the culture medium obtained from the abluminal compartment after 48 h exposure. More specifically, about 50% and 70% of the initial IC₁₀ and IC₃₀ DON doses from the luminal compartment were respectively

Table 2

Combination Index (CI) values of DON-ZEA binary mixture (as function of concentration combination) on the different co-culture systems cells after 48 h exposure, calculated according to the Response Additivity (CI_{RA}) and Independent Joint Action (CI_{IJA}).

Mycotoxin concentration		CI _{RA}	CI _{IJA}
DON-ZEA IC10	Co-culture A	NA	NA
	Co-culture B	NA	NA
	Co-culture C	NA	NA
	Co-culture D	NA	NA
DON-ZEA IC ₃₀	Co-culture A	0.78 (Syn)	0.74 (Syn)
	Co-culture B	0.64 (Syn)	0.63 (Syn)
	Co-culture C	0.92 (Add)	0.88 (Syn)
	Co-culture D	NA	NA

 $CI<1,\,0.9\leq CI\leq1.1$ and CI>1.1 indicate synergism (Syn), additivity (Add) and antagonism (Ant), respectively; NA = Non applicable as no significant cytotoxicity was observed.

quantified in the abluminal compartment. For ZEA, less than 10% of the initial doses were measured in the abluminal part, indicating a possible uptake and/or biotransformation of this mycotoxin by Caco-2 and/or HepaRG cells. On the contrary, for bi-culture B, only ZEA was measured in the abluminal fraction of this culture system. Indeed, less than 10% of the initial DON doses were measured in the culture medium after 48 h exposure, while about 23% of the initial ZEA IC₃₀ dose was quantified (and only 10% of the IC₁₀ dose). Concerning bi-culture C, both toxins were detected in the abluminal compartment. More precisely, about 17% of the initial IC₁₀ doses and 100% of the initial IC₃₀ doses for both mycotoxins were quantified in the abluminal compartment with THP-1 cells.

4. Discussion

Nowadays, numerous toxicological studies are carried out on in vitro experimental models in order to reduce animal experimentation which represents an important ethical challenge. Since the 3R principle (Replace, Reduce, Refine) was established, immense efforts have been made to improve these cell culture models so that they may serve as a substitute for animal experiments. Nevertheless, most of these in vitro studies are conducted on an individual cell type, and therefore do not accurately reflect the complexity of in vivo conditions. More specifically, this type of culture does not take into account some major parameters required for appropriate cell and tissue physiology reproduction, including cell to cell communication. To better mimic an in vivo situation, sophisticated in vitro models have been developed, namely 3D culture systems, including multicellular spheroid systems (Kelm and Fussenegger, 2004), and more recently, multi-tissue organon-a-chip platforms [31]. Nonetheless, the complexity and the cost of such systems might discourage their use. Thus, simple co-culture systems could represent an interesting alternative to conventional culture models.

In the present study, we investigated the individual and combined cytotoxic effects of DON and ZEA on different human cell lines cultured in bi- and tri-culture systems at low and moderate cytotoxicity levels (namely, using IC_{10} and IC_{30}). We determined these concentrations on Caco-2 cells (in the present study, supplementary data) and previously on HepaRG cells [35] in mono-culture experiments. IC values highlighted a similar sensitivity to DON and ZEA for the two cell lineages.

Results from bi-culture A suggested possible uptake and/or biotransformation of ZEA by the Caco-2 and/or HepaRG cells since only a small fraction of the initial concentration was detected by Q-TOF LC-MS analysis in the abluminal compartment after 48 h exposure (for both tested ZEA concentrations). These results seemed to confirm the study by Videmann et al. [40] showing that Caco-2 cells rapidly produced the reduced and conjugated forms of ZEA and that they easily crossed the cell membranes. On the contrary, the significant fraction of DON in the abluminal compartment implied that this mycotoxin was poorly metabolized by Caco-2 and/or HepaRG cells. These results were consistent with the observed HepaRG cell mortality level (i.e. 20% at the highest tested concentration) after 48 h DON exposure and the absence of cytotoxicity after ZEA exposure. These findings seemed to be correlated with data from literature indicating that ZEA is mainly metabolized by hepatocytes and small intestinal epithelial cells following ingestion and absorption, while DON is generally poorly metabolized after intake. To clarify this aspect, it would be of interest in the future to quantify ZEA metabolites in the abluminal compartment. To date, two major biotransformation pathways of ZEA have been described: i) hydroxylation of ZEA resulting in the phase-I-metabolites α -zearalenol (α -ZEL) and β zearalenol (β -ZEL) - which can be further reduced to α -zearalanol (α -ZAL) and β -zearalanol (β -ZAL), respectively - and ii) conjugation of ZEA and its reduced forms with glucuronic acid and sulfate leading to the phase-II-conjugation products [16]; [21]. This was also recently confirmed using the human Caco-2 cell line [26]. However, there are significant differences in the metabolic conversion of ZEA among



Fig. 3. Trans-endothelial electric resistance (TEER) measurement made across Caco-2 cells in different co-culture combinations after 48 h exposure to DON, ZEA and DON + ZEA at their respective IC_{10} and IC_{30} (mean \pm SEM fold of control of TEER measured using an epithelial voltohmmeter, N = 3) * = TEER measured mean significantly different from negative control (arbitrarily set to 1) (P < 0.05).

animal species [17]. In human, α -ZEL metabolite has been described as the most preponderant metabolite followed by β-zearalenol using Caco-2 cells [40]. Regarding DON, as for ZEA, its absorption, elimination and metabolism differ in the tested animal species. Nevertheless, in various species exposed to DON, the major metabolite found in urine and stools is de-epoxy-DON (DOM-1) [46]. This less toxic metabolite is produced via intestinal or rumen microorganims rather than by the liver or other organs [24]. In humans, very little data are available on the toxicokinetics of DON. Pestka and Smolinski [23] reported that human drug-metabolizing enzymes such as human CYP450 enzymes do not detoxify DON into DOM-1. However, in the liver, DON can be conjugated to glucoronides leading to the formation of a non-toxic detoxification product, glucuronide conjugated-DON (DON-GLU) [45]. Only unmetabolized DON as well as DON-GLU were detected from human urine samples analysis [20]. Furthermore, DON metabolite profiles in humans is region-dependent [10,39,41-43].

Regarding bi-culture B, in contrast to bi-culture A, only ZEA was detected in the abluminal compartment with THP-1 cells and consistently induced a significant cytotoxic effect towards this cell lineage after 48 h (i.e. 25% for the highest tested concentration). Thus, these results suggested that, in the bi-culture system A, ZEA that easily passed the cell barrier, was mainly absorbed and/or metabolized by HepaRG cells rather than by Caco-2 cells. Indeed, ZEA was only found in the abluminal compartment in bi-culture B while both bi-cultures A and B had Caco-2 cells in the luminal compartment. For DON, this mycotoxin was present in the abluminal fraction of bi-culture A but was not detected in bi-culture B. Previous data showed that DON could be partially transported across the intestinal epithelium by simple diffusion via the paracellular route, through the tight junctions [29]. So, two hypothesizes could explain this result: DON might be metabolized by i) THP-1 cells or ii) Caco-2 cells after being "stimulated" by the immune cells as they were in contact with the abluminal medium. Indeed, THP-1 cells are able to secrete cytokines and chemokines to recruit other cells, and therefore can interact with other cell lines in co-culture systems [4]. For example, it was found that close proximity of THP-1 cells and adipocytes induced insulin resistance and apoptosis in the latter cell lineage [14]. Conversely [36], observed that adipocytes secreted soluble factors which promoted polarization of THP-1 macrophages

Table 3

Measured concentrations of DON and ZEA in the abluminal compartment after 48 h incubation. Results are expressed as the mean \pm SEM of concentrations from 3 independent experiments quantified using Q-TOF LC-MS, and the corresponding mycotoxin proportion (%) is indicated in brackets.

Co-culture		А	В	С
DON IC ₁₀ (DON mono-exposure)	Incubation concentration (µM)	1.6	1.6	0.2
	Final concentration (48 h) (µM)	0.23 ± 0.02 (50.3%)	0 ± 0 (0%)	0.01 ± 0.01 (17.5%)
DON IC ₃₀ (DON mono-exposure)	Incubation concentration (µM)	3	3	2.3
	Final concentration (48 h) (µM)	0.63 ± 0.05 (73.3%)	0.09 ± 0.04 (10.5%)	0.71 ± 0.09 (100%)
ZEA IC ₁₀ (ZEA mono-exposure)	Incubation concentration (µM)	24	24	20 µM
	Final concentration (48 h) (µM)	0.41 ± 0.11 (6%)	0.68 ± 0.3 (9.9%)	0.78 ± 0.14 (13.7%)
ZEA IC ₃₀ (ZEA mono-exposure)	Incubation concentration (µM)	31	31	33
	Final concentration (48 h) (µM)	0.66 ± 0.09 (7.5%)	2.04 ± 0.41 (23%)	9.96 ± 1.27 (100%)
DON IC_{10} (DON + ZEA co-exposure)	Incubation concentration (µM)	1.6	1.6	0.2
	Final concentration (48 h) (µM)	0.25 ± 0.01 (54.7%)	0.01 ± 0.01 (1.1%)	0.01 ± 0.01 (17.5%)
DON IC_{30} (DON + ZEA co-exposure)	Incubation concentration (µM)	3	3	2.3
	Final concentration (48 h) (µM)	0.64 ± 0.01 (74.7%)	0 ± 0 (0%)	0.68 ± 0.10 (100%)
ZEA IC_{10} (DON + ZEA co-exposure)	Incubation concentration (µM)	24	24	20
	Final concentration (48 h) (µM)	0.47 ± 0.09 (6.9%)	0.7 ± 0.21 (10.2%)	$1.02 \pm 0.17 (17.9\%)$
ZEA IC_{30} (DON + ZEA co-exposure)	Incubation concentration (µM)	31	31	33
	Final concentration (48 h) (µM)	0.71 ± 0.1 (8%)	2.1 ± 0.41 (23.7%)	9.46 ± 2.7 (100%)

The incubation concentrations correspond to the mycotoxin concentrations added in the luminal compartment while the final concentrations correspond to those measured in the abluminal compartment after 48 h incubation.

and, similarly, Kolesar et al. [15] reported that the cytokine gene expression of THP-1 monocytes could be regulated by chemokine secretion of respiratory epithelial A549 cells. In addition, it is well-known that, in vivo, intestinal epithelial cells are regulated by the immune cells located in the intestinal epithelium via soluble factors [30]. Different studies reported that immune cells such as lymphocytes, macrophages and dendritic cells can affect the intestinal epithelial cells via secretion of soluble factors such as cytokines, including the tumor necrosis factor- α (TNF- α) and interferon- γ (INF- γ) in response to stimulation [19,28,44]. More specifically, the co-culture of THP-1 macrophages and Caco-2 cells resulted in an increase of lactate dehydrogenase release from the latter and a decrease in the TEER of the monolaver indicating that co-culturing with THP-1 induced cell damage to Caco-2 cells [28,44]. On the other hand, although biotransformation reactions for converting xenobiotics and drugs into hydrophilic metabolites occur mainly in the liver (which expresses the most prominent class of biotransformation enzymes), Edling et al. [7,8] highlighted the ability of THP-1 cells to metabolize some hepatotoxic drug compounds.

In bi-culture system C, both DON and ZEA were detected in the abluminal compartment with THP-1 cells, highlighting that this immune cell line did not absorb or metabolize DON as previously hypothesized. Indeed, the entire doses used for exposure experiments for both toxins were found in the abluminal fractions after 48 h exposure (for the highest tested concentration of DON and ZEA). Moreover, we assumed earlier that ZEA was mainly absorbed and/or metabolized by HepaRG cells. Nevertheless, bi-culture C conditions showed that ZEA was still present in the abluminal compartment. Thus we might hypothesize that THP-1 monocytes affect the hepatic cells, preventing ZEA biotransformation by these cells. In addition, we can also assume that the HepaRG cell monolayer did not constitute an efficient cell barrier, and had a higher permeability than the Caco-2 cell monolayer. Furthermore, only ZEA seemed to induce significant cell mortality on THP-1 cells (i.e. 20% at the highest tested concentration, while no significant cell mortality was observed for DON). Thus, it could be hypothesized that DON was in the abluminal fraction since a short. As it is well-established that mycotoxin toxicity increases with exposure time, it would be interesting to assess THP-1 cell viability after a longer exposure duration to verify that this mycotoxin exerts its toxicity on this cell line (which is normally the case at such a concentration [34]).

Interestingly, for all bi-culture systems, the combined use of DON + ZEA at the highest tested concentration induced significant cytotoxicity on the cell line cultured in the abluminal compartment. More specifically, in bi-cultures A and B, cell viability was reduced by about 40% for HepaRG and THP-1 cells, while a decrease in 25% was observed for THP-1 cells in bi-culture C. Thus, the mixture of these two mycotoxins led to an increased cytotoxicity as only 20% of cell mortality was observed after DON exposure and no significant cytotoxicity was noticed after ZEA exposure in bi-culture A, while the opposite was observed in bi-cultures B and C. Nevertheless, the combined cytotoxic effect was characterized by slight synergism in bi-culture C (0.85 \leq C \leq I0.9), whereas it was described as moderate synergism in bi-culture B (0.7 \leq CI = 0.85) and synergism in bi-culture A $(0.3 \le CI = 0.7)$ as suggested by Chou [6]. In bi-cultures A and B, only one mycotoxin was mostly present in the abluminal compartment (DON in bi-culture A and ZEA in bi-culture B) while both mycotoxins were significantly present in the abluminal section in the bi-culture system C. This could be explained by the different degree of synergism between the different co-culture systems, as cytotoxic combined effects of mycotoxins are partly dose-dependent [33].

Cell integrity was also assessed using TEER values as they reflect tight junctions functionality and are therefore accepted as strong indicators of cellular barrier integrity [37]. Here, we observed a significant TEER fall in both bi-culture systems A and B, and this for all treatments, except after DON exposure in bi-culture B. This indicated that the Caco-2 cell monolayer was sensitive to mycotoxins, mostly when co-cultured with THP-1, which might be explained by the potential cytokine release from these cells. It was previously reported that DON affected the TEER of the Caco-2 cell monolayer and could potentially trigger intestinal inflammation [13,27,29]. On the contrary, some studies described the lack of TEER changes when the Caco-2 cell monolayer was exposed to ZEA, suggesting that the cell monolayer permeability was affected by some soluble factors expressed by HepaRG and THP-1 cells in the present study. Thus, it would be of interest to monitor such factors in the culture medium.

Finally, cell viability was assessed in the tri-culture conditions and showed that neither DON nor ZEA, alone and in combination, induced significant cytotoxicity on the THP-1 cell lineage. Thus, these findings highlighted that, by increasing cell numbers and therefore cell to cell interactions, the mycotoxin cytotoxic effect decreased.

In conclusion, in the present study we observed that the cytotoxic effects of DON and ZEA were strongly impacted by cell to cell interactions. More specifically, the toxicological impact of these mycotoxins decreased with increasing co-culture model complexity. It is worth mentioning that the simultaneous exposure to DON and ZEA may be more toxic than the presence of these mycotoxins alone as shown by the higher cytotoxicity of the mixture in the different bi-culture models. Overall, the obtained results highlighted the fact that our proposed biand tri-culture models can be used to gain further knowledge on the toxicological impact of mycotoxins, used individually or in combination, on different cell lines. More particularly, these simple and innovative cell culture systems could be useful to study the toxicity of acetylated forms of DON present in cereals, namely 3-acetyl and 15acetyldeoxynivalenol, that have been found to be deacetylated by the small intestine and liver using ex vivo models based on human explants [57]. Finally, the use of these cell lines in a differentiated state would also be of interest to get closer to in vivo conditions.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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Transparency document

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Supplementary data

Table S1: Q-TOF LC-MS parameters to detect and quantify the studied analytes. DON: deoxynivalenol, ZEA: zearalenone, MW: molecular weight, RT: retention time, m/z: mass to charge ratio, ESI: electrospray ionisation

Compound -	MW	RT	FCI	Quantifier Ion	Qualifier Ion	D ²	
Formula	(g/mol)	(min)	LSI	(m/z)	(m/z)	К	
DON - C15H20O6	296.1259	1.3	+	[M+Na] ⁺ 319.1151	[M+H] ⁺ 297.1328	0.99	
ZEA – C18H22O5	318.1467	13.2	-	[M-H] ⁻ 317.1392	n/a	0.99	



Figure S1: Cytotoxic effect of DON and ZEA on Caco-2 cells after 48 h exposure in mono-culture experiments (percentage of cell viability quantified using MTS bioassay, N=3). Dose-effect curves were generated using Microsoft TableCurve 2D Software v.5.01.

Les points marquants de cette étude sont les suivants :

- Les effets cytotoxiques du DON et de la ZEA sur les cellules situées au fond des puits varient en fonction du type cellulaire avec lequel elles sont cocultivées.
- La combinaison DON+ZEA, à la plus forte concentration testée, induit une cytotoxicité significativement plus importante que le DON et la ZEA présents individuellement dans 3 des 4 systèmes de coculture (effet synergique).
- Les dosages des mycotoxines dans les compartiments abluminaux montrent de grandes différences d'un système de coculture à l'autre, suggérant de potentielles interactions entre les différents types cellulaires qui pourraient modifier leur réponse face à l'exposition aux mycotoxines.
- Les résultats de cytotoxicité sur la triculture suggèrent que la cytotoxicté des mycotoxines diminue quand la complexité du système (c'est-à-dire le nombre de modèles cellulaires cocultivés) augmente.

La figure 14 schématise de façon synthétique les principaux résultats obtenus dans cette étude.



Figure 14 : Représentation schématique des principaux effets biologiques observés dans l'étude n°5 pour les doses CI₃₀.

Chapitre III : DISCUSSION, CONCLUSION ET PERSPECTIVES

Dans le premier chapitre de cette thèse, il est souligné que le risque lié à l'exposition aux mycotoxines constitue un problème de santé publique d'actualité encore mal caractérisé. En particulier, de nombreuses interrogations persistent sur les effets des expositions multiples aux mycotoxines, cas le plus fréquent pourtant, ainsi que sur ceux des faibles concentrations d'exposition appliquées sur le moyen, voire le long terme. De plus, la législation en vigueur en Europe visant à limiter l'utilisation d'animaux pour les expériences scientifiques (2010/63/UE) conduisent à rechercher des modèles cellulaires humains pertinents et adaptés, représentatifs des cellules in vivo dont ils dérivent. Dans le domaine de la toxicologie, certaines hypothèses demandent à être confirmées ou écartées, notamment le caractère perturbateur endocrinien (de la ZEA par exemple) ou le potentiel cancérigène de certaines mycotoxines, telles que les TCTs, aujourd'hui classées dans le groupe 3 par le CIRC (c'est-àdire inclassables quant à leur cancérogénicité pour l'Homme du fait du manque de données pour pouvoir statuer). D'autre part, l'identification de biomarqueurs d'effet ou d'exposition, aujourd'hui devenue possible grâce aux méthodes à haut débit (transcriptomique, protéomique, métabolomique), est fortement attendue et pourrait, le cas échéant, apporter de nouveaux indicateurs dans l'appréciation épidémiologique des expositions et des risques encourus par les consommateurs (Galtier et al., 2006).

Dans ce contexte, ce projet de thèse avait différents objectifs :

- Etudier les effets d'une multi-exposition aux mycotoxines afin de mieux caractériser l'incidence de la présence simultanée des mycotoxines fréquemment rencontrées dans l'alimentation. Ceci a été réalisé sur des modèles cellulaires humains pertinents, à la fois avec une exposition à court terme (toxicité aiguë) mais également à plus long terme (toxicité chronique).
- Etudier plus finement les mécanismes cellulaires impliqués dans la réponse des cellules à ces expositions. Pour cela, l'étude de l'expression de gènes ciblés ainsi que l'étude du protéome des cellules ont été réalisées.

Les différents modèles cellulaires utilisés dans ce projet sont les monocytes THP-1, les cellules hépatiques HepaRG et les cellules intestinales Caco-2, tous indifférenciés. Cependant, les HepaRG ont été majoritairement utilisés, aussi bien pour évaluer les effets d'une exposition répétée et prolongée aux fusariotoxines, que pour les analyses mécanistiques non ciblées, car ils constituent un modèle original, métaboliquement stable et compétent.

Partie I : Cytotoxicité aiguë des fusariotoxines, seules et en mélange

Dans ce projet de thèse, les monocytes THP-1 et les hépatoblastes HepaRG, ont été choisis pour étudier les effets toxicologiques de six fusariotoxines seules et en mélange (pour rappel, il s'agissait de DON, MON, FB1, ZEA, NIV et T2 ainsi que des mélanges DON+MON, DON+FB1, DON+ZEA et NIV+T2). Quant aux cellules intestinales Caco-2, elles ont été utilisées uniquement lors de la dernière étude en coculture dans laquelle les effets du DON et de la ZEA ont été évalués, seuls et en mélange. Pour chacun de ces modèles cellulaires, la viabilité cellulaire suite à l'exposition individuelle et combinée aux fusariotoxines a été le premier paramètre étudié. Leurs effets cytotoxiques ont donc été comparés en fonction du modèle cellulaire considéré.

1. Cytotoxicité individuelle des fusariotoxines

Les résultats issus des expositions individuelles aux fusariotoxines montrent que ces toxines affectent les deux types cellulaires, THP-1 et HepaRG, dans le même ordre, à savoir : T2 > NIV > DON > ZEA, FB1, MON. Cependant, les THP-1 semblent, de manière générale, plus sensibles à ces toxines que les cellules HepaRG. En effet, la comparaison des CI₅₀ indique que les valeurs obtenues pour les THP-1 sont entre 1,5 et 30 fois plus faibles que pour les HepaRG selon la fusariotoxine étudiée (tableau 7). Autrement dit, des doses plus importantes sont nécessaires dans le cas des HepaRG pour induire une même mortalité chez les deux lignées. Par ailleurs, les Caco-2 ont montré une sensibilité plus importante que les HepaRG à la ZEA, mais une sensibilité similaire au DON. La faible sensibilité des cellules hépatiques aux mycotoxines par rapport aux monocytes peut s'expliquer par le fait que le foie est l'organe de détoxification et doit donc être capable de supporter des expositions à des xénobiotiques. En particulier, la capacité des cellules hépatiques à métaboliser de nombreuses mycotoxines grâce, notamment, aux différents cytochromes P450 qu'ils expriment, pourrait justifier cette différence de sensibilité. De plus, de nombreuses mycotoxines, dont les TCTs, sont connues pour être de puissants immuno-modulateurs (Bennett and Klich, 2003; Bouaziz et al., 2013). Différentes études ont également relevé une plus forte sensibilité des cellules issues du système immunitaire par rapport à d'autres lignées cellulaires. Par exemple, Nielsen et al. (2009) ont comparé les CI₅₀ obtenues après 48h sur 9 lignées cellulaires d'origine humaine (cellules d'hépatome Hep-G2, cellules de carcinome du larynx Hep-2, du côlon Caco-2 et des poumons A459, cellules de rhadbomyosarcome A204, monocytes U937, lymphocytes-B RPMI 8226 et lymphocytes-TH1 Jurkat ainsi que des cellules primaires

endothéliales de cordon ombilical HUVEC) pour différents TCTs (tels que T2, HT2, DON et NIV), et ont observé que les lignées les plus sensibles étaient celles ayant une origine immunitaire (lymphocytes et monocytes). De plus, ces auteurs ont relevé pour toutes les lignées cellulaires une sensibilité plus importante pour la T2, suivie du NIV et du DON, comme nous avons pu nous-mêmes l'observer sur les THP-1 et les HepaRG. Une revue de 2014 (Cheli et al., 2014) soulignait d'importants écarts de sensibilité aux mycotoxines entre les différents modèles cellulaires étudiés comme le montre la large gamme de valeurs des CI₅₀ recensées (tableau 7). On s'aperçoit alors que les CI₅₀ obtenues pour les THP-1 et les HepaRG se situent, de manière générale, dans la limite basse des CI₅₀ déjà recensées (à l'exception de la CI₅₀ observée pour la T2 chez les HepaRG). Néanmoins, les valeurs des CI₅₀ rapportées par Cheli et al. (2014) l'ont été indépendamment du temps d'exposition et du test utilisé.

Tableau	7:	Comparaison	des	CI50	obtenues	pour	Τ2,	NIV,	DON,	ZEA,	FB1	et	MON	sur
différents	mo	dèles cellulair	es.											

Mycotoxine	CI ₅₀ des THP-1	CI ₅₀ des HepaRG	CI ₅₀ des Caco-2	Gamme des CI ₅₀ recensées		
	(cette étude)	(cette étude)	(cette étude)	(Cheli et al., 2014)		
T2	0,0058 µM	0,19 µM	-	0,003 - 0,23 μM		
NIV	0,77 μM	2,84 µM	-	0,16 - 17,75 μM		
DON	1,82 µM	7,35 μM	> 10 µM	0,4 - 125 μM		
ZEA	36,31 µM	55,12 µM	54,01 µM	26 - 313 μM		
FB1	> 10 µM	> 10 µM	-	16,5 - 900 μM		
MON	> 10 µM	$> 10 \mu M$	-	403 - 1020 μM		

Les tests les plus couramment employés pour l'évaluation de la viabilité cellulaire sont les tests aux sels de tétrazolium (MTT, MTS et WST), le test à la résazurine (Alamar Blue), le test de fixation du rouge neutre (Neutral Red) ou encore la mesure de la libération de la lactate déshydrogénase (LDH) dans le milieu de culture. Ils permettent une estimation quantitative de l'activité métabolique des cellules ou de leur intégrité membranaire. Différentes études ont cependant souligné que, selon le test choisi pour estimer la cytotoxicité d'une toxine, les résultats peuvent varier de manière significative (Bony et al., 2006; Fotakis and Timbrell, 2006; Petty et al., 1995). De plus, la sensibilité d'un test peut varier en fonction du modèle cellulaire considéré. Par conséquent, puisqu'aucune recommandation n'existe à ce jour pour l'évaluation de la cytotoxicité des mycotoxines, le paramètre de viabilité cellulaire à mesurer devrait être choisi en fonction de sa sensibilité maximale pour la lignée cellulaire considérée (Cheli et al., 2014).

2. Cytotoxicité combinée des fusariotoxines

L'étude des co-expositions aux fusariotoxines révèle des effets cytotoxiques combinés majoritairement antagonistes sur les THP-1 pour les quatre mélanges étudiés. En revanche, pour les HepaRG, des effets essentiellement synergiques ont été observés sur la réduction de la viabilité cellulaire. Seul le mélange NIV+T2 dans le cas des cellules hépatiques induit de l'antagonisme pour certaines doses testées (tableau 8).

Tableau 8 : Résumé des effets cytotoxiques combinés des couples DON+MON, DON+FB1, DON+ZEA et NIV+T2 sur les THP-1 et les HepaRG selon les modèles de l'additivité (Weber et al., 2005), de Bliss (Bliss, 1939) et Chou-Talalay (Chou, 2006; Chou and Talalay, 1984) tels qu'observés au cours de cette étude.

Couple Méthode TH		THP-1	HepaRG				
	Additivitá	Antagonista	Synergique puis additif à la plus forte				
DON+MON	Additivite	Antagoniste	dose				
	Blice	Antagoniste	Synergique puis additif à la plus forte				
	D1135	Antagoniste	dose				
	Additivitá	Antagoniste	Synergique puis additif à la plus forte				
	7 Idulti vite	Anugoinste	dose				
DON+FB1		Antagoniste aux plus faibles					
	Bliss	doses puis additif aux doses	Synergique				
		supérieures					
	A dditivitá	Antagoniste	Synergique aux plus faibles doses puis				
	7 tuanti vite	Anagomste	antagoniste aux doses supérieures				
	Blice	Antagoniste	Synergique puis additif à la plus forte				
DUNTLEA	D1135	Antagoniste	dose				
	Chou-	Antagoniste puis synergique	Synergique				
	Talalay	à la plus forte dose	Syncigique				
	Additivité	Antagoniste	Synergique à la plus faible dose puis				
	7 taanii vite	Anagomste	antagoniste aux doses supérieures				
NIV+T2		Antagoniste aux plus faibles	Synergique à la plus faible dose puis				
	Bliss	doses puis additif aux doses	additif aux doses supérieures				
		supérieures	additif aux doses superiories				
	Chou	Synergique à la plus faible	Synergique à la plus faible dose,				
	Talalay	dose et antagoniste aux doses	antagoniste aux doses intermédiaires,				
	raiaray	supérieures	puis synergique de nouveau				

Ces dernières années, de plus en plus d'études se sont intéressées aux effets combinés des mycotoxines, la viabilité étant l'un des paramètres les plus largement étudiés. Dans la revue présentée en introduction (Smith et al., 2016), il est souligné que les effets combinés cytotoxiques de ces toxines varient considérablement en fonction du modèle cellulaire étudié, mais aussi selon la durée d'exposition choisie et la concentration testée. D'autres paramètres peuvent également influencer les effets, tels que l'état de différenciation des cellules ou encore la composition du milieu de culture (avec ou sans SVF par exemple). Dans la plupart des cas, les effets toxicologiques des mélanges ne peuvent pas être prédits uniquement sur la base des effets individuels des mycotoxines, et le meilleur moyen de connaître les effets d'un mélange reste encore de le tester expérimentalement (Gao et al., 2016; Grenier and Oswald, 2011).

Il existe aujourd'hui différentes stratégies pour évaluer les effets combinés des mycotoxines, la plus couramment utilisée étant celle de l'additivité des réponses (« response additivity ») utilisée par Weber et al. (2005). Il en existe cependant beaucoup d'autres, telles que le modèle d'indépendance des actions proposé par Bliss (« Independence Joint Action ») (Bliss, 1939), celui de l'additivité des doses défini par Loewe (« concentration addition ») (Loewe and Muischnek, 1926) et la méthode de Chou et Talalay (Combination Index-Isobologram) (Chou, 2006; Chou and Talalay, 1984). Ces approches ont été récemment décrites par Foucquier and Guedj (2015). Elles permettent de caractériser les interactions des mycotoxines lorsqu'elles sont présentes ensemble dans le mélange, mais pas d'élucider les mécanismes par lesquels ces interactions sont produites. Quelques auteurs ont comparé certaines de ces méthodes entre elles pour l'analyse de leurs résultats de co-exposition aux mycotoxines. Ainsi, Tammer et al. (2007) ont pu observer des effets synergiques avec le modèle de Bliss et de l'antagonisme avec celui de Loewe. Nous avons nous-mêmes pu constater, sur les HepaRG, des différences en fonction du modèle mathématique utilisé (tableau 8). Bien que l'approche proposée par Chou et Talalay soit de plus en plus employée, elle présente, comme tous les autres modèles, certaines limites. Par exemple, cette méthode est inutilisable lorsque la courbe effet-dose ne peut pas être générée ou est difficile à modéliser (comme cela a pu être le cas dans notre étude pour la MON et la FB1). De plus, cette méthode ne peut être utilisée que sur des courbes effet-dose monotones. Par conséquent, les concentrations élevées pour chacune des toxines du mélange, qui étaient déjà exclues pour l'ajustement de la courbe sigmoïde, sont exclues de l'analyse IC. Il est également essentiel d'inclure uniquement la NOAEC (no observed adverse effect concentration, concentration sans effet néfaste observé)

et d'exclure des mesures inférieures à cette concentration sans effet, car ces données fausseraient la conformité de la loi de masse. Une autre condition préalable est la conformité de l'ensemble de données avec la loi de masse sur laquelle repose l'équation d'effet médian, qui peut simplement être vérifiée *via* le coefficient de corrélation (r) (devant idéalement être supérieur ou égal à 0,95 (Chou, 2010)). Ainsi, tant qu'il n'y aura pas de méthodologie de référence, Foucquier and Guedj (2015) suggèrent l'utilisation simultanée de différentes approches pour l'analyse des combinaisons de mycotoxines.

2.1. Effet combiné antagoniste

Une des hypothèses les plus probables pour expliquer l'effet antagoniste de différentes substances toxiques présentes dans un mélange est leur capacité à entrer en compétition pour se lier au(x) même(s) récepteur(s) cellulaire(s), et que l'une d'entre elles présente une plus grande affinité pour ce(s) récepteur(s). Cette compétition provoquerait alors l'accumulation des autres composés du mélange et induirait ainsi une toxicité globale inférieure à celle prédite sur la base de l'additivité des effets individuels (Alassane-Kpembi et al., 2015; Wang et al., 2014). Ce serait le cas du DON avec le MON, la FB1 et la ZEA, ainsi que du NIV en présence de la T2 une fois absorbés par les THP-1 (le DON étant plus toxique que le MON, la FB1 et la ZEA, et la T2 plus toxique que le NIV). Cette hypothèse est d'ailleurs appuyée par les résultats de l'expression des MAPKs qui montrent l'activation de ces voies de signalisation par toutes les fusariotoxines étudiées et suggérant des cibles communes à ces toxines. Il aurait également pu être intéressant de compléter cette étude en testant des inhibiteurs de ces voies de signalisation afin de valider leur implication directe dans la mort des cellules et, dans le cas contraire, identifier les autres voies impliquées. De plus, l'étude de l'expression de marqueurs de l'inflammation cellulaire (cytokines ou dérivés oxygénés par exemple) connus pour être modulés par certaines mycotoxines serait également pertinente dans ce type d'étude, ainsi que l'expression de certains récepteurs tels que les TLR (Toll-like receptors).

D'autres hypothèses peuvent expliquer un effet combiné antagoniste : i) l'une des toxines empêche l'entrée de l'autre ou ii) altère son élimination/ dégradation (ou la stimule dans le cas d'un métabolite plus toxique que le composé parent). L'étude des effets des mycotoxines seules et en cocktail sur l'expression des systèmes d'efflux (tels que la P-glycoprotéine) et de dégradation (CYP450 par exemple) pourrait apporter des élements de réponse concernant le mécanisme à l'origine des interactions entre les mycotxines d'un mélange.

D'autre part, comme nous avons pu l'observer, la perte de certains marqueurs de surface spécifiques aux monocytes après exposition aux différentes toxines et mélanges peut suggérer que les cellules sont possiblement devenues non fonctionnelles. L'expression des cytokines (qui est un événement lié à la réponse inflammatoire des cellules) aurait alors pu être mesurée, ainsi que la différentiation des monocytes en macrophages.

2.2. Effet combiné synergique

Concernant le synergisme d'une combinaison de substances toxiques, il peut être expliqué par une action « facilitée » de ces mycotoxines, signifiant que l'action secondaire de l'une des toxines augmente l'activité de l'autre composé du mélange, ou « complémentaire », lorsque les composés agissent sur la même cible mais sur des sites différents ou qui se superposent, ou encore sur des cibles différentes mais de la même voie biologique (Jia et al., 2009). Ce serait le cas des fusariotoxines étudiées une fois absorbées par les HepaRG. Une action d'ailleurs très différente du mélange DON+ZEA par rapport au DON et à la ZEA individuellement a pu être observée à l'échelle moléculaire, en particulier sur l'expression de certains gènes codant pour des fonctions spécifiques des hépatocytes, mais aussi sur le protéome global des HepaRG.

2.3. Effet combiné additif

Seul le couple NIV+T2 a révélé des effets cytotoxiques additifs pour plusieurs doses testées sur les deux types cellulaires (*via* le modèle de Bliss). Cette additivité pourrait être due à un mécanisme d'action similaire aux TCTs, basé sur la similarité de structure de ces deux composés, conduisant à la mort cellulaire, sans toutefois qu'il y ait d'interférences entre les deux toxines. En effet, l'additivité est représentative d'une absence d'interaction entre les différents composés d'un mélange.

2.4. Effet combiné dose-dépendant

D'autre part, comme nous avons pu nous-mêmes l'observer, différentes études ont montré que les effets combinés des mycotoxines pouvaient varier en fonction des concentrations testées. Par exemple, Gao et al. (2016) ont observé des effets synergiques du couple AFM1+ZEA sur la viabilité cellulaire des Caco-2 après 24h d'exposition à faibles doses, et antagonistes à plus fortes doses, alors qu'ils ont constaté l'exact opposé pour le mélange AFM1+OTA. Les auteurs suggèrent que l'antagonisme observé à faibles doses pour ce deuxième mélange peut

s'expliquer par la compétition des deux mycotoxines pour le glutathion dans les cellules, alors qu'à des concentrations plus importantes, l'AFM1 et l'OTA peuvent être incorporées plus facilement dans les membranes cellulaires grâce à leur structure lipophile, et ainsi exercer leur toxicité de façon synergique. Sur les HepaRG, nous avons pu essentiellement observer du synergisme à faibles doses et de l'antagonisme à plus fortes doses (en particulier pour les mélanges DON+ZEA et NIV+T2). Une des hypothèses pouvant expliquer cette action dosedépendante serait la capacité de ces mycotoxines à cibler plusieurs récepteurs cellulaires, dont certains en commun. Ainsi, à faibles doses, la fixation de chacune des mycotoxines sur leurs cibles respectives entraînerait une action « complémentaire » de celles-ci, conduisant à une synergie sur la mortalité cellulaire alors, qu'à plus fortes doses, ces mycotoxines entreraient en compétition pour les récepteurs qu'elles ont en commun, provoquant cette fois de l'antagonisme sur la mortalité des cellules.

2.5. Effet combiné temps-dépendant

Par ailleurs, le temps d'exposition peut également influencer le type d'interaction entre les mycotoxines d'un même mélange. En effet, certaines études ont reporté des effets synergiques à 24h et antagonistes à 72h ou inversement en fonction des mélanges étudiés (Gao et al., 2016; Juan-García et al., 2016; Tatay et al., 2014). Bien qu'il soit difficile d'expliquer les mécanismes à l'origine des effets cytotoxiques combinés des mycotoxines à différents temps d'exposition, on peut supposer que la toxicité observée serait liée aux métabolites de ces toxines obtenues à des temps d'expositions suffisamment longs. En effet, Tatay et al. (2014) ont reporté un taux de biotransformation de ZEA allant de 4% après 24h à 81% après 48h d'exposition dans les cultures de cellules ovariennes de hamster CHO-K1. De même, dans les cultures d'hépatocytes humains HepG2, Juan-García et al. (2016) ont observé que 63% du 3-ADON initialement ajouté au milieu était encore présent après 24h d'exposition, et seulement 44% après 72h.

3. Mécanismes de mort cellulaire impliqués dans la cytotoxicité des fusariotoxines

La comparaison des mécanismes de mort cellulaire induits par les fusariotoxines sur les monocytes et les hépatoblastes souligne certaines similitudes pour les deux modèles cellulaires. En effet, pour les deux lignées, l'analyse par le double marquage annexine V/iodure de propidium montre que le DON, la MON et le NIV ainsi que le mélange DON+MON, ne semblent pas induire d'apoptose et/ou de nécrose à la concentration testée

(CI₅₀) et aux temps d'exposition sélectionnés (3, 6, 12 et 18h). De plus, la FB1 et la ZEA, seules et en mélange avec le DON, provoquent des phénomènes d'apoptose et de nécrose des deux lignées cellulaires. Néanmoins, les mélanges DON+FB1 et DON+ZEA induisent ces mécanismes de mort cellulaire dès 3h pour les monocytes et seulement après 18h pour les cellules hépatiques. Enfin, pour la T2 et le mélange NIV+T2, l'induction de la nécrose a pu être observée dès 12h d'exposition chez les THP-1 alors qu'elle n'est pas significative chez les HepaRG, même après 18h. Ainsi, la comparaison de ces deux modèles montre que, bien qu'il existe une réponse propre à chaque type cellulaire, des similitudes sur certains mécanismes peuvent être observés pour une mycotoxine ou un mélange considéré, soulignant des effets toxicologiques inhérents à chaque mycotoxine (ou mélange).

Partie II : Hépatotoxicité aiguë et chronique des fusariotoxines à différents niveaux moléculaires

Les mécanismes cellulaires impliqués dans la toxicité des mycotoxines sont encore mal caractérisés pour la plupart de ces toxines. En effet, la plus grande partie des études s'intéressant aux effets toxicologiques des mycotoxines se concentrent essentiellement sur les effets cytotoxiques de celles-ci, et lorsque les mécanismes sont étudiés, il s'agit bien souvent d'approches ciblées. D'un point de vue moléculaire, toute exposition à un composé toxique peut induire des modifications de l'intégrité cellulaire, de la fonctionnalité de l'ADN (au niveau génomique) et de l'expression au niveau des ARN (transcriptomique), des protéines (protéomique) et des métabolites (métabolomique) dues à des interactions moléculaires entre la substance toxique et la cible biologique. L'étude précise de ces mécanismes moléculaires et de la façon dont ils affectent l'homéostasie cellulaire, en particulier par les approches « omiques », est donc nécessaire à la compréhension approfondie des mécanismes de toxicité des mycotoxines. C'est pourquoi, dans un second temps, nous nous sommes intéressés à la réponse des cellules HepaRG exposées au couple DON+ZEA (ainsi qu'au DON et à la ZEA seuls) à cette échelle « omique ». En particulier, suite à une courte exposition au DON et/ou à la ZEA (1h), nous avons, dans un premier temps, mesuré la quantité d'ARNm codant pour certains facteurs spécifiques du foie et, dans un second temps, effectué une analyse du protéome global des hépatoblastes. Enfin, nous avons recherché si des expositions répétées à plus long terme (exposition chronique sur 14, 28 et 42 jours) à de faibles concentrations en DON et ZEA, individuellement ou en mélange, pouvaient mettre en évidence des effets différents de ceux observés après des expositions aiguës (sur un temps d'1h). Nous avons donc mesuré la viabilité des cellules ainsi que les niveaux d'expression des gènes codant pour certaines fonctions spécifiques des hépatocytes (les mêmes que pour l'étude sur 1h).

1. Effets toxicologiques des fusariotoxines sur l'expression génique et protéique

L'étude ciblée de l'expression de certains gènes montre que la réponse des HepaRG après 1h d'exposition au mélange DON+ZEA est très différente de celle observée pour les toxines seules. En effet, aux CI₁₀ (obtenues après 48h d'exposition par le test MTS), le DON et la ZEA induisent peu de changements sur les niveaux d'expression des gènes codant pour des fonctions importantes des hépatocytes (telles que les CYP 4F3B et 3A4 de la famille P450, ou encore l'albumine, la transferrine et l'adolase B). En revanche, le mélange impacte beaucoup plus leur expression. En effet, cinq des sept gènes étudiés codant pour ces facteurs hépatiques

voient leur expression augmenter par rapport au témoin, pouvant indiquer une « hyperactivité » des cellules. Néanmoins, l'interprétation des résultats de l'expression des gènes s'appuit uniquement sur des différences de significativité (P<0,05) entre le contrôle (cellules non exposées) et les cellules exposées au mycotoxines. Certains auteurs considèrent qu'un gène est sous- ou sur-exprimé s'il présente une expression au moins 2 fois plus faible ou importante que celle observée pour le contrôle. Ici, en tenant compte de la significativité et de ce facteur 2, seul le mélange DON+ZEA induierait un changement d'expression, et ce sur le gène codant pour le CYP3A4, également sur-exprimé au niveau de la protéine. Cette surexpression suggère une dégradation plus importante de ces toxines (essentiellement de la ZEA connue pour avoir une dégradation médiée par les CYP) lorsqu'elles sont en mélange. Il aurait donc été intéressant de doser leurs métabolites afin de valider cette hypothèse. De plus, certains métabolites de la ZEA étant connus pour être plus toxiques que la ZEA, telles que l'α-zéaralénol, cela pourrait expliquer le synergisme observé après 48h sur la mortalité cellulaire. Par ailleurs, il est à noter que la mesure l'expression du CYP3A7, qui est la forme fœtale du CYP3A4, aurait été particulèrement inréressante puisque les cellules étudiées dans cette étude sont non différenciées. D'un autre côté, l'analyse du protéome global des cellules (toujours après 1h d'exposition à ces toxines et aux CI₁₀) montre également une réponse plus importante pour le mélange que pour les toxines seules, avec beaucoup plus de protéines ayant une abondance diminuée par rapport au témoin. En revanche, il ne semble pas y avoir de corrélation entre les variations observées par qPCR sur l'expression de certains gènes codant pour les facteurs ciblés et les modifications mesurées par LC-MS/MS sur les niveaux d'abondance de ces mêmes facteurs à l'échelle du protéome. Il convient toutefois de noter que les analyses d'ARNm et de protéines ont été effectuées au même instant (1h) ; par conséquent, il serait intéressant d'effectuer la cinétique des ARNm étudiés et des protéines correspondantes afin d'avoir une meilleure vue de leur régulation connexe aux niveaux transcriptionnel et traductionnel. En effet, il a pu être souligné qu'il existait un décalage spatial et temporel entre le transcription et la traduction dans les cellules (Berg et al., 2002). D'autres études ont également souligné des différences entre les niveaux d'ARNm mesurés et ceux des protéines correspondantes. Par exemple, Graziani et al. (2015) ont observé que le DON augmentait de manière dose-dépendante l'ARNm de l'oxide nitrique synthase inductible dans les cellules Caco-2, alors que l'abondance de cette protéine ne changeait pas car sa dégradation était stimulée par son ubiquitinylation. Les effets sur le niveau d'abondance des protéines ne sont donc pas directement corrélés à la production d'ARNm puisque leur régulation respective ne se produit pas nécessairement simultanément après

exposition de la cellule à un toxique. De plus, les toxines peuvent également induire des modifications traductionnelles et/ou post-traductionnelles des protéines, ainsi que leur dégradation (Edfors et al., 2016; Vogel and Marcotte, 2012). Cela expliquerait donc, en plus du décalage spatial et temporel entre la transcription et la traduction, que l'on ne retrouve pas les mêmes fonctions impactées au niveau du gène et de la protéine dans notre étude après 1h d'exposition aux mycotoxines. D'autre part, il serait également pertinent de tester davantage de temps d'exposition courts, car certaines mycotoxines induisent une réponse transitoire avec activation de certains gènes entre 0 et 12 h puis absence de réponse jusque 12, 48 ou 96 h. Des cinétiques permettraient ainsi d'expliquer les effets très différents observés entre 1 et 24 h d'exposition avec les analyses protéomiques, et de tirer des conclusions plus fines. Pour compléter ces résultats, il serait intéressant d'analyser le métabolome de ces cellules après avoir été exposées au DON et/ou à la ZEA. En effet, contrairement aux gènes et aux protéines dont la fonction nette est soumise à une régulation épigénétique et/ou à une modification posttraductionnelle, les métabolites sont les produits finaux des processus de régulation cellulaire (ce qui inclue les produits de dégradation des mycotoxines qui pourraient renseigner sur les enzymes impliquées), et sont étroitement corrélés au phénotype du système biologique étudié (Fiehn, 2002; Halama, 2014). Une récente revue suggère d'ailleurs d'étudier la toxicologie des mycotoxines d'un point de vue holistique, en développant des approches « multiomiques » combinant à la fois l'étude du génome, du transcriptome, du protéome et du métabolome d'un système biologique comme outil pour mieux comprendre les mécanismes d'action sous-jacents aux effets toxiques connues des mycotoxines (Dellafiora and Dall'Asta, 2017).

D'autre part, il serait également intéressant de réaliser des études similaires sur des cellules différenciées, c'est-à-dire des hépatocytes matures, et de comparer les résultats avec ceux obtenus pour les hépatoblastes. En effet, l'état de différenciation des cellules a une incidence sur leur sensibilité à ces toxines, les cellules différenciées étant généralement plus résistantes que celles indifférenciées. Ceci s'explique par l'expression plus importante de certaines enzymes impliquées dans la dégradation des xénobiotiques telles que les CYP chez les cellules différenciées (avec toutefois l'exception des aflatoxines dont la dégradation CYP-dépendante génère un métabolite plus toxique que le composé parent).

Par ailleurs, lors de l'étude du protéome global des HepaRG après 1h et 24h d'exposition aux mycotoxines, des dosages de ces mycotoxines dans les surnageants de culture ont été réalisés

à la fin des périodes d'incubation. Les résultats soulignent qu'à 1h, aucune des deux toxines ne semble pas avoir été significativement absorbées par les cellules, puisqu'il n'y a pas de différence mesurable entre les concentrations intiales et finales. En revanche, après 24h, alors que seules des traces de ZEA sont retrouvées dans le surnageant, des quantités identiques au contrôle sont mesurées pour le DON. Pourtant, une réponse cellulaire significative est observée pour les deux mycotoxines au niveau du protéome, indiquant qu'une partie du DON est rentrée dans la cellule et a pu interagir physiquement avec les ribosomes. Une étude a également montré que, in vitro, le DON n'était accumulé ni dans les cellules épithéliales humaines du rein, ni au niveau des fibroblastes pulmonaires humains (Königs et al., 2007). En particulier, les auteurs n'ont mesuré de différences significatives entre les concentrations retrouvées en fin d'exposition et celles ajoutées initialement pour le DON dans les surnageants de culture des deux types cellulaires, aussi bien après 48h que 120h d'exposition, avec seulement de très faibles quantités intra-cellulaires retrouvées. De plus, DOM-1 ainsi que DON-GLU ont également été recherchés dans les surnageants, mais aucune trace n'a pu être détectée pour ces deux métabolites. Il aurait alors était intéressant de vérifier dans notre étude les concentrations intracellulaires en DON, potentiellement non-nulles. De plus, des contrôles sans cellules, dans lesquels serait mesurée la concentration en mycotoxine retrouvée dans le milieu de culture après différents temps d'incubation, permettraient de déterminer la proportion de mycotoxine réellement dissoute dans le milieu et celle liée au plastique du flacon de culture par exemple. En effet, la liposolubilité d'une subtance toxique étudiée in *vitro* a une incidence sur sa solubilité dans le milieu et donc sur les effets observés.

2. Effets toxicologiques aigus et chroniques des fusariotoxines

Après une exposition prolongée et répétée des HepaRG au DON et/ou à la ZEA visant à se rapprocher des conditions d'exposition associées à l'ingestion d'aliments contaminés, les effets sur les niveaux d'expression des gènes codant pour certains facteurs spécifiques de ces cellules apparaissent très différents de ceux observés en conditions aiguës (après seulement 1h d'exposition). En effet, après 1h, les toxines seules n'induisent que peu de changements sur l'expression des gènes étudiés alors qu'après 14 jours, de nombreux changements sont observés. Il est toutefois important de noter que les doses testées en chronique sont bien moins cytotoxiques que la CI₁₀ utilisée pour l'étude sur 1h car, bien que très faiblement cytotoxique après 48h (c'est-à-dire 10% de mortalité), la CI₁₀ a provoqué la mortalité de la totalité des cellules après plusieurs jours d'exposition, indiquant que la cytotoxicité de ces

toxines augmente avec le temps d'exposition (accumulation des effets dû à une exposition prolongée). Plus particulièrement, après 14 jours d'exposition, à la plus faible dose testée (DM), tous les traitements (DON, ZEA et DON+ZEA) semblent induire des changements significatifs sur l'expression des gènes étudiés, en provoquant notamment une « hyperactivité » des HepaRG (ZEA induisant plus de changements que DON et le mélange). Cependant, après 28 jours, DON et ZEA seuls induisent beaucoup moins de changements sur l'expression de ces gènes, suggérant une possible adaptation des cellules à ces toxines. En revanche, concernant le mélange, après 28 jours, l'expression des gènes apparaît davantage impactée qu'après 14 jours, avec cette fois une diminution des taux des ARNm associés aux facteurs étudiés, et notamment ceux des CYP 3A4 et 4F3B, soulignant une activité métabolique réduite des HepaRG. Pourtant, à cette dose (DM), aussi bien après 14 que 28 ou 42 jours, aucune réduction de la viabilité cellulaire n'a été observée, quel que soit la mycotoxine ou le mélange testé. Dans la mesure où les deux enzymes CYP3A4 et CYP4F3B sont impliquées dans la dégradation de nombreux substrats, il est possible que le mélange DON+ZEA puisse altérer la réponse à ces substrats en réduisant leur métabolisme. Leur toxicité sera alors possiblement différente selon qu'elle s'exprime avant ou après biotransformation de ces substrats. Ainsi, des interactions entre les deux mycotoxines peuvent intervenir et engendrer des effets toxiques non prévisibles à long terme. Par ailleurs, le phénomène d'adaptation de l'organisme à une exposition répétée aux mycotoxines a déjà pu être observé in vivo chez les poulets de chair nourris plusieurs semaines avec des aliments contaminés avec l'AFB1 (Yunus et al., 2011). Dans cette dernière étude, à de faibles doses (0,07 mg d'AFB1/kg), les auteurs ont observé une diminution temporaire des performances des volailles, notamment une prise de poids plus faible par rapport au témoin, pendant la troisième semaine d'exposition seulement. Après la quatrième semaine, plus aucun effet significatif n'a été observé. Dans cette même étude, les auteurs ont pu mettre en évidence la capacité de l'intestin en tant qu'organe dynamique à s'adapter à l'exposition chronique à l'AFB1.

Ainsi, les premiers résultats obtenus sur les HepaRG après une exposition répétée et prolongée au DON et à la ZEA constituent un argument pertinent pour la poursuite de telles études qui pourraient éclairer sur les effets et mécanismes d'action de chaque mycotoxine ainsi que sur leurs interactions potentielles suite à une exposition chronique, plus représentative des conditions réelles d'exposition du consommateur. Néanmoins, il convient de souligner qu'il est très difficile, voire impossible, de mimer la situation d'exposition *in*

vivo de manière réaliste puisque celle-ci correspond à une exposition simultanée à de nombreux contaminants (aussi bien alimentaires qu'environnementaux) et que la composition ainsi que les quantités relatives peuvent évoluer au cours du temps pour chaque individu. D'autre part, les concentrations utilisées dans cette étude en mol/L ont été extrapolées à partir de doses exprimées en µg/kg pc/jour (pour la DM et la DJT) et en µg/kg d'aliment (pour la TM). Pour ces différentes doses, il a été considéré que 1kg de poids corporel et que 1kg d'aliment correspondaient à 1L de milieu de culture. Ainsi, pour la DM et la DJT, cela revient à dire qu'un individu de 70 kg mangerait tous les deux jours l'équivalent de son poids en céréales. Il aurait alors été intéressant de prendre la quantité moyenne de céréales ingérée par jour pour un adulte (soit environ 30 g en France) et de la rapporter à la quantité totale d'aliment ingérée quotidiennement (environ 2,5 kg) pour utiliser ce ratio et le rapporter au volume de milieu de culture (soit 1,2% du milieu de culture contaminé). Néanmoins, aucune cytotoxicité n'ayant été observée à ces doses (DM et DJT) même après 42 jours d'exposition, on peut en déduire que de plus faibles doses n'auraient pas non plus affecté la viabilité cellulaire. De plus, il est très compliqué d'extrapoler de telles doses en concentrations à tester sur des cultures in vitro, ce qui explique la rareté des données sur des expositions chroniques in vitro. D'un autre côté, pour la TM, comme la quantité de milieu de culture est proportionnelle au nombre de cellules cultivées (tout comme la quantité de céréales ingérée est proportionnelle à la taille et au poids d'un individu), en ajoutant la TM dans le volume total de culture, cela revient à mimer un individu qui ne mangerait que des céréales contaminées à la TM autorisée par la commission européenne, sans tenir compte des données de toxicocinétiques (rares chez l'Homme). Ainsi, les résultats obtenus à cette concentration, notamment sur la viabilité cellulaire, sont à prendre avec du recul, comme souligné dans l'article.

Partie III : Systèmes de culture cellulaire *in vitro* pour l'évaluation de la toxicité des mycotoxines

Dans une démarche éthique visant à réduire l'expérimentation animale, de nombreuses études de toxicologie sont aujourd'hui menées sur des systèmes *in vitro*. En particulier, depuis l'instauration de la règle des « 3R » (Réduire, Raffiner, Remplacer), d'importants efforts ont été faits pour améliorer ces modèles de culture cellulaire afin qu'ils puissent remplacer les expériences animales. L'utilisation d'un modèle *in vitro* précis pour les études de toxicité doit permettre l'identification des voies et mécanismes cellulaires affectés tout en reflétant au mieux la situation *in vivo*. Un tel système doit également permettre une culture fonctionnelle à long terme des cellules. Or, la plupart de ces études sont réalisées dans des systèmes de culture classiques 2D faisant intervenir un seul type cellulaire, et ne reflètent donc pas la complexité d'un organe 3D, entraînant alors des divergences entre les données expérimentales *in vitro*. En particulier, ce type de culture ne prend pas en compte certains facteurs importants permettant de reproduire avec précision la physiologie des cellules et des tissus, tels que la communication entre la cellule et sa matrice ainsi que la communication entre cellules, mais aussi l'exposition consécutive de plusieurs types cellulaires lors de l'ingestion d'un xénobiotique.

Les systèmes de coculture *in vitro* permettent de mieux mimer la situation *in vivo* par rapport aux cultures *in vitro* classiques en tenant compte des interactions entre différents types cellulaires, et représentent donc une alternative intéressante aux systèmes traditionnels. Quelques auteurs ont d'ailleurs déjà mis en place de tels systèmes de culture comprenant des macrophages THP-1 avec des adipocytes (Spencer et al., 2010), des cellules intestinales (Girón-Calle et al., 2010; Hayashi et al., 2012; Ishimoto et al., 2011; Jiang et al., 2013; Kolesar et al., 2012; Moyes et al., 2010; Watanabe et al., 2004), des lymphocytes T (Azenabor et al., 2011; Kanwar and Kanwar, 2009) ou encore des cellules musculaires lisses de vaisseaux (Li et al., 2006; Zhang et al., 2008). Il a pu, par exemple, être montré que l'expression des cytokines par les monocytes THP-1 pouvait être régulée par la sécrétion de chimiokines par les cellules alvéolaires A549 (Kolesar et al., 2012). Réciproquement, différentes études ont souligné la capacité des cellules immunitaires (telles que les lymphocytes, les macrophages et les cellules dendritiques) à affecter la réponse des cellules de l'épithélium intestinal *via* la sécrétion de facteurs solubles tels que les cytokines (McKay and Singh, 1997; Satsu et al., 2006; Watanabe et al., 2004). Ainsi, il est ressorti de ces études que la coculture de cellules THP-1 et de cellules issus de tissus spécifiques pouvait être utilisée comme modèle pour mimer la chimiotaxie in vivo (Chanput et al., 2014). Un des résultats majeurs obtenus lors de nos cocultures est l'important écart observé pour les concentrations mesurées en DON et ZEA dans les compartiments abluminaux entre les différents systèmes de coculture. Plus particulièrement, entre les deux cocultures faisant intervenir les Caco-2 cultivées dans les inserts, des quantités plus importantes en DON ont été retrouvées après 48h dans les compartiments abluminaux en présence des HepaRG que des THP-1, alors que le contraire a été observé pour la ZEA. Ces résultats interrogent alors sur l'existence de potentielles interactions entre les différents types cellulaires qui pourraient modifier la réponse de ces cellules face à l'exposition aux mycotoxines. Un autre résultat intéressant est l'absence de cytotoxicité sur les THP-1 dans le système de triculture, suggérant que l'impact toxicologique des mycotoxines diminue lorsque le nombre de modèles cellulaires cocultivés augmente. De plus, il serait par la suite pertinent de s'intéresser à d'autres paramètres cellulaires que la viabilité, et notamment l'expression de certains CYP ou encore celle des P-glycoprotéines, qui pourrait expliquer le synergisme observé avec le mélange. Le dosage des métabolites du DON et de la ZEA dans les milieux de culture ainsi que de certains facteurs solubles sécrétés par les cellules (tels que des cytokines) pourraient apporter des éléments de réponse nécessaires à la compréhension du rôle de chaque type cellulaire dans de tels systèmes de coculture. Il serait également intéressant de doser les mycotoxines et leurs métabolites dans les compartiments abluminaux de cultures « contrôles », dans lesquelles ne seraient cultivées qu'un seul type cellulaire dans les inserts, afin d'évaluer dans quelle mesure la réponse cellulaire peut être modifiée lors de la coculture. Enfin, il est à noter que la plupart des concentrations mesurées dans les compartiments abluminaux pour le DON et la ZEA après 48h (quelle que soit la coculture considérée) sont cohérentes avec la cytotoxicité observée (par comparaison avec les courbes effet-dose obtenues dans nos études pour le DON et la ZEA sur les THP-1 et les HepaRG individuellement après 48h). Par exemple, dans la coculture (i) (Caco-2/HepaRG), 22% de mortalité cellulaire a été mesurée sur les HepaRG après 48h d'exposition au DON (CI_{30}) pour une concentration retrouvée de 0,63 µM, ce qui correspond bien au pourcentage de mortalité provoqué par une telle concentration sur les HepaRG en monoculture. Toutefois, une exception a pu être relevée : dans la coculture (iii) (HepaRG/THP-1), l'exposition au DON (CI₃₀) n'a pas provoqué de cytotoxicité de façon significative sur les THP-1 alors qu'une concentration importante en DON (0,71 µM) a été retrouvée. Une telle concentration aurait dû normalement induire environ 40% de mortalité cellulaire. C'est pourquoi, il serait également

pertinent de réaliser des tests de cytotoxicité sur des temps d'exposition plus longs afin de pouvoir espérer voir les effets cytotoxiques de cette mycotoxine normalement provoqués à de telles doses. De plus, les dosages des mycotoxines pourraient également être réalisés à différents temps d'incubation afin d'évaluer le temps nécessaire à ces toxines pour traverser les barrières cellulaires (Caco-2 ou HepaRG). Par ailleurs, il est important de noter que, dans le but de pouvoir comparer les résultats obtenus en co-culture avec ceux des mono-cultures, toutes les cellules ont été utilisées dans un état indifférencié, c'est-à-dire à l'état d'entérocytes immatures pour les Caco-2, à celui de monocytes pour les THP-1 et en tant qu'hépatoblastes pour les HepaRG. En effet, la différenciation des cellules impacte leur sensibilité aux mycotoxines. Il aurait été particulièrement intéressant de développer des systèmes de co-culture semblables à ceux utilisés dans ce projet avec des cellules, cette fois, différenciées. En particulier, afin de se rapprocher des conditions *in vivo*, un système de tri-culture faisant intervenir des entérocytes matures cultivés dans les inserts en contact avec des macrophages, situés sous ces inserts, ainsi qu'avec des hépatocytes situés au fond des puits, serait pertinent pour étudier la toxicité des mycotoxines et de leurs métabolites.

Bien que les cocultures permettent de surmonter certains inconvénients des systèmes 2D classiques (en particulier la communication intercellulaire), ils sont encore loin de reproduire avec précision toutes les fonctions cellulaires observées dans un tissu. Les systèmes de culture 3D *in vitro* semblent être des modèles pertinents pour tenter de se rapprocher des conditions in vivo. En particulier, des systèmes sphéroïdes multicellulaires ont été développés et sont aujourd'hui réalisables à haut débit (Kelm and Fussenegger, 2004). Dans ce type de système, le nombre de cellules et par conséquent la taille du sphéroïde sont ajustables. Une étude menée sur les HepaRG soulignait que les sphéroïdes d'HepaRG 3D avaient une meilleure fonctionnalité que les cultures d'HepaRG 2D classiques et représentaient donc un bon modèle pour l'étude de la toxicité de médicaments, en particulier pour l'étude de leur métabolisme (Mueller et al., 2014). De la même façon, Ramaiahgari et al. (2014) ont montré que les sphéroïdes 3D d'HepG2 présentaient une meilleure fonctionnalité par rapport aux monocouches. De plus, les auteurs ont pu maintenir ce système dans des plaques de 384 puits pendant 28 jours et en ont conclu que ce modèle présentait une meilleure sensibilité pour l'identification des composés hépatotoxiques. Dans une étude plus récente encore, Ramaiahgari et al. (2017) ont pu tirer les mêmes conclusions quant aux sphéroïdes 3D d'HepaRG. Par ailleurs, ce système apparaît adapté aux techniques de coculture, et semble donc être un modèle particulièrement prometteur pour les études de toxicologie (Haycock,

2011). Il existe donc aujourd'hui tout un champ d'investigation possible pour mimer plus finement le contexte *in vivo*, et qui pourrait permettre une évaluation plus précise du risque « mycotoxines » pour la santé humaine et animale.

Le tableau 9 synthétise tous les résultats obtenus, au cours de ce travail de thèse, avec le mélange DON+ZEA. Il souligne la variabilité des résultats en fonction du modèle cellulaire étudié, de la concentration testée, de la durée d'exposition choisie, de l'approche sélectionnée ainsi que des interactions entre cellules.

De manière plus globale, au cours de notre vie, nous pouvons être exposés à de multiples subtances chimiques provenant de sources diverses. Ainsi, l'autorité européenne de sécurité des aliments (European Food Safety Authority, EFSA) a récemment entrepris de développer des méthodes permettant d'évaluer les risques associés à la problématique complexe des mélanges dans la chaîne alimentaire. L'étude du comportement de subtances chimiques combinées est une question extrêmement complexe, avec un nombre de combinaisons potentiellement infini. L'objectif premier de cette initiative de l'EFSA est d'élaborer de nouvelles méthodes pour harmoniser les méthodologies d'évaluation des risques liés à l'exposition multiple aux contaminants de la chaîne alimentaire et de développer des outils scientifiques qui permettront de modéliser leur toxicité combinée. Un premier rapport vient d'être publié souligant l'ampleur de ce récent projet (EFSA, 2017).

Tableau 9 : Tableau récapitulatif des différents résultats obtenus pour le mélange DON+ZEA dans ce travail de thèse.	
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Durée d'ernosition	1h	3h	6h	12h	18h	24h	48h	14 jours	28 jours	42 jours
u exposition	DON : 0,06 μM ZEA : 1,8 μM		DON : 1,8 ZEA : 36,	3 μM 3μM		DON : 0,1- 10µM ; ZEA : 2-100 µM				
Monoculture THP-1	Activation MAPKs p38, ERK1/2 et SAPK/JUNK	Apoptose	Apoptose associée à y potentiel transmembranaire mitochondrial	Apoptose et nécrose + perte des marqueurs de surface cellulaire CD14 et CD71	Apoptose et nécrose	Pas de données	Effet cytotoxique antagoniste	Pas de données	Pas de données	Pas de données
	DON : 0,2 μM ZEA : 20 μM		DON : 7,3 ZEA : 55,	5 μM 1μM		DON : 0,2 μM ZEA : 20 μM	DON : 0,2- 10μM ; ZEA : 1,5-75 μM	2	DON : 1,6 nM et 2,5 μM ZEA : 0,085 nM et 0,24 μM	
Monoculture HepaRG	Approche ciblée : ↗ niveaux ARNm pour CYP3A4, CYP4F3B, transferrine, albumine et aldolase B + ↗ expression protéine CYP3A4 mais ¥ claudine-1	Ni	Ni apoptose, ni	Apontose et	Apoptose	Approche non ciblée : 39 protéines avec abondance modifiée (dont 2⊅ et	Effet	Forte cytotoxicité observée sur une exposition répétée tous les 2 jours à la plus forte concentration	Forte cytotoxicité observée sur une exposition répétée tous les 2 jours à la plus forte concentration	Forte cytotoxicité observée sur une exposition répétée tous les 2 jours à la plus forte concentration
	Approche non ciblée : 35 protéines avec abondance modifiée (dont 67 et 292) → impact sur l'organisation chromosomique, le métabolisme de l'ADN et le cycle cellulaire	apoptose, ni nécrose	nécrose	nécrose	et nécrose	37¥) → impact sur régulation de l'activité hydrolase et de l'expression des gènes	cytotoxique synergique	A la plus faible dose, peu de modifications sur les niveaux d'expression des genes et protéines ciblés (=fonctions spécifiques des hépatocytes)	A la plus faible dose, IJ des niveaux d'ARNm pour tous les gènes ciblés ainsi que les protéines associées (=fonctions spécifiques des hépatocytes)	
Biculture Caco-2							DON : 3μM ZEA : 31 μM			
HepaRG	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	Effet cytotoxique synergique sur les HepaRG	Pas de données	Pas de données	Pas de données
Biculture Caco-2 THP-1	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	DON : 3μM ZEA : 31 μM Effet cytotoxique synergique sur les THP-1	Pas de données	Pas de données	Pas de données
Biculture HepaRG THP-1	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	DON : 2,3μM ZEA : 33 μM Effet cytotoxique additif sur les THP-1	Pas de données	Pas de données	Pas de données
Triculture Caco-2 HepaRG THP-1	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	Pas de données	DON : 3μM ZEA : 31 μM Effet cytotoxique additif sur les THP-1	Pas de données	Pas de données	Pas de données

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Annexes

Annexe I : Valorisation de la recherche

Publications

Publications acceptées

- Smith, M.-C.; Madec, S.; Coton, E.; Hymery, N.; 2016. Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins* 8(4), 94. https://doi.org/10.3390/toxins8040094
- Smith, M.-C.; Madec, S.; Troadec, S.; Coton, E.; Hymery, N.; 2017. Effects of fusariotoxin co-exposure on THP-1 human immune cells. *Cell Biology and Toxicology* 1-15. https://doi.org/10.1007/s10565-017-9408-7
- Smith, M.-C.; Madec, S.; Pawtowski, A.; Coton, E.; Hymery, N.; 2017. Individual and combined toxicological effects of deoxynivalenol and zearalenone on human hepatocytes in *in vitro* chronic exposure conditions. *Toxicology Letters* 280: 238-246. https://doi.org/10.1016/j.toxlet.2017.08.080
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- Smith, M.-C.; Timmins-Schiffman, E.; Coton, M.; Coton, E.; Hymery, N.; Nunn, B.; Madec, S.; 2018. Differential impacts of individual and combined exposures of deoxynivalenol and zearalenone on the HepaRG human hepatic cell proteome. *Journal of Proteomics* 173: 89-98. https://doi.org/10.1016/j.jprot.2017.11.025
- Smith, M.-C.; Gheux, A.; Coton, M.; Madec, S.; Hymery, N.; Coton, E., 2018. In vitro coculture models to evaluate acute cytotoxicity of individual and combined mycotoxin exposures on Caco-2, THP-1 and HepaRG on the human cell lines. Chemico-Biological Interactions 281: 51-59. https://doi.org/10.1016/j.cbi.2017.12.004

Publications en préparation

Smith, M.-C.; Coton, E.; Hymery, N. Influence of multiwell plate type on cell viability assessment in *in vitro* toxicity studies.

Communications orales

- Smith, M.-C.; Madec, S.; Troadec, S., Coton, E.; Hymery, Effects of fusariotoxin coexposure on THP-1 human immune cells, *Journées Mycotoxines*, Toulouse, France, 15 et 16 mars 2016
- Smith, M.-C.; Hymery, N.; Timmins-Schiffman, E.; Nunn, B.; Coton, E.; Madec, S., Incidence of acute and chronic co-exposure of fusariotoxins on human hepatocytes, *World Mycotoxin Forum 2016*, Winnipeg, Canada, 6-9 juin 2016
- Smith, M.-C.; Hymery, N.; Timmins-Schiffman, E.; Nunn, B.; Coton, M.; Coton, E.; Madec, S. Early proteomic changes induced by fusariotoxin single and combined exposures on human hepatocytes, 7th Euro Global Summit on Clinical Microbiology and Mycotoxins, Amsterdam, Pays-Bas, 27 et 28 février 2017

Communications affichées

Smith, M.-C.; Madec, S.; Coton, E.; Hymery. Incidence sur les cellules humaines de la coexposition aux fusariotoxines, 5^{ème} journée des doctorants de l'ED SICMA, Brest, France, 24 septembre 2015

Journées Mycotoxines

Toulouse - 15 & 16 mars 2016

Effet de l'exposition de cellules immunitaires humaines THP-1 à des mélanges de fusariotoxines

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Les céréales représentent la ressource la plus importante de l'alimentation humaine et animale au monde. Parmi les microorganismes contaminant les céréales, certaines espèces de champignons (Aspergillus, Fusarium et Penicillium) sont toxinogènes. En France, les Fusarium sont les espèces les plus problématiques en raison de leur prévalence, leur écologie, leur physiologie et de la large gamme de mycotoxines (appelées fusariotoxines) produites. Trois familles de fusariotoxines sont particulièrement importantes en raison de leur grande toxicité et de leur incidence dans les produits agricoles européens : les trichothécènes (essentiellement la déoxynivalénol, la nivalénol et la toxine T-2), les fumonisines et la zéaralénone. La gravité des effets dépend de la durée d'exposition, des doses et des combinaisons de toxines ingérées. Les espèces de Fusarium sont capables de produire simultanément différentes mycotoxines ; de plus, les matrices peuvent être contaminées par plusieurs espèces de champignons à la fois. En France, 91 % des échantillons céréaliers analysés en 2013 étaient multi-contaminés. Cependant, le risque associé à cette multicontamination est, à l'heure actuelle, pas ou peu étudié. Dans ce contexte, notre étude a pour objectifs i) de mieux caractériser le risque induit par l'exposition simultanée de cellules humaines à des fusariotoxines et ii) d'étudier les mécanismes cellulaires impliqués dans la réponse à l'exposition à une ou plusieurs mycotoxines, via des approches toxicologiques et protéomiques. Pour cela, nous avons défini quatre couples de fusariotoxines fréquemment retrouvés dans l'alimentation, et évalués leur cytotoxicité in vitro sur la lignée monocytaire humaine THP-1. En particulier, nous nous sommes intéressés aux effets sur la viabilité et la mortalité cellulaire, ainsi que sur l'expression et l'activation de protéines de voies de signalisation impliquées dans la réponse au stress des cellules, telles que les MAP kinases (ERK1/2, P38 et SAPK/JNK). Après 48h d'exposition, les CI₅₀ obtenues pour les toxines T-2, nivalénol, déoxynivalénol et zéaralénone sont respectivement de 0,006, 0,8, 1,8 and 36 µM. Seules les CI₂₀ et CI₃₀ ont été obtenues pour la fumonisine B1 et la moniliformine à la concentration testée la plus élevée (10 µM). Les effets cytotoxiques observés en co-exposition sont principalement antagonistes. L'étude des mécanismes impliqués dans la mort cellulaire montrent des effets apoptotiques et nécrotiques des mélanges. Enfin, l'étude des voies de signalisation impliquées dans la réponse au stress des cellules suite à l'exposition aux mycotoxines, montre une activation des formes phosphorylées ERK1/2, SAPK/JNK et p38 dès 1h d'exposition à de faibles doses cytotoxiques (CI_{10}).



Incidence of acute and chronic coexposure of fusariotoxins on human hepatocytes

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Cereals are the most important food and feed resource in the world. Climatic and agricultural practices changes observed over the last years, including the reduction of fungicide use, could lead to food safety problems, especially concerning microbiological contaminations. Among microorganisms contaminating grains, some fungal species (Aspergillus, Fusarium and Penicillium) are toxigenic. In northern temperate regions of the world (America, Asia and Europe), Fusarium spp. are the most problematic species due to their prevalence, ecology, physiology and wide range of mycotoxins (called fusariotoxins) produced. Three fusariotoxins families are particularly important because of their high toxicity and their occurrence in European agricultural products: trichothecenes (mainly deoxynivalenol, nivalenol and T-2 toxin), fumonisins and zearalenone. The severity of the effects depends on the time of exposure, doses and mycotoxin combinations. Fusarium species are able to produce several mycotoxins simultaneously. Moreover, matrices can be simultaneously contaminated by different fungi species. Worldwide, 47% of the cereal samples analyzed in 2013 were multi-contaminated. However, the risk associated with this multi-contamination is no or a little studied for the moment, whereas there could have implications in various fields including regulatory. Indeed, to this date, mycotoxin regulation has been established for each mycotoxin considered individually. In this context, our main objectives were: (1) to better characterize the risk induced by the simultaneous presence of Fusarium toxins in acute and chronic exposure conditions, and (2) to study the cellular mechanisms involved in the response to the exposure to one or more mycotoxins through toxicology and proteomic approaches. For this purpose, we defined 4 fusariotoxin binary mixtures and evaluated their in vitro toxicity on the human hepatocyte cell line: HeparG. After 48 h of exposure, IC₅₀ for toxin T-2, nivalenol, deoxynivalenol and zearalenone were 0.2, 2.8, 5.9 and 55 µM, respectively. Only IC₁₀ were obtained at 10 µM for fumonisin B1 and moniliformin. Coexposure observed effects were mainly synergistics or additives. Concerning chronic exposure, we selected 3 exposure times (14, 30 and 90 days) and three subtoxic concentrations corresponding to the average exposure dose of French adult population, the tolerable daily intake established by the JECFA and the maximum level permitted in cereals by the European regulation. Effects on viability and cytochrome expression were evaluated. Very few data exist on acute and chronic human mycotoxin co-exposure. Yet, it appears that chronic exposure to multicontamination in mycotoxin is an important future challenge in risk assessment.

7th Euro Global Summit on Clinical Microbiology and Mycotoxins

February 27-28, 2017 Amsterdam, Netherlands

Early proteomic changes induced by fusariotoxin single and combined exposures on human hepatocytes

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While the reality of mycotoxin co-contamination of food commodities is now wellestablished, the assessment of the toxicological impact of mycotoxin mixtures is still rare. Moreover, studies concerning the mechanistic cellular response to mycotoxins (alone or in mixture) are lacking. Among the infinite number of possible mycotoxin mixtures found, combinations of toxins from Fusarium spp. (called fusariotoxins) are particularly widespread in the North temperate zone of the world and therefore of interest. In this context, our main objective was to compare the cellular mechanisms involved in the response to single and combined exposures of the human hepatocyte cell line HepaRG to two relevant fusariotoxins, deoxynivalenol and zearalenone. After 1h of exposure with deoxynivalenol and/or zearalenone at low cytotoxic doses (IC10), proteomes of HepaRG cells were analysed by LC-MS/MS and compard to the control condition without mycotoxin. Among the 3000 identified proteins per sample, 55 showed a significant enhanced or reduced abundance compared to the non exposed cells. Interestingly, none of these 55 proteins were in common between the cells exposed to deoxynivalenol and those exposed to zearalenone. Noteworthy, very few proteins were common between the mixture and the toxins alone. Cells exposed to deoxynivalenol showed an increased expression of proteins involved in DNA topological changes, chromosome segregation and proteolysis, whereas zearalenone mainly induced changes for proteins involved in the response to steroid hormone stimulus. Concerning the mixture, the main affected biological processes were, among others, cell cycle phase, DNA packaging and cell division. Thus, these results highlighted that different cellular pathways responded to the different single and combined mycotoxin exposures.

Annexe II : Données supplémentaires pour l'étude n°3

UNIPROT accession	Protein name	Fold change ¹	ZSstatistic ¹	UNIPROT accession	Protein name	Fold change ¹	ZSstatistic ¹
DON – 1 h				DON – 24 h			
P22061	Protein-L-isoaspartate(D-aspartate) O- methyltransferase	0.79	2.15	Q99598	Translin-associated protein X	0.976	2.3049
Q5VYK3	Proteasome-associated protein ECM29 homolog	0.735	2.4447	O00186	Syntaxin-binding protein 3	0.903	2.2747
Q9GZZ9	Ubiquitin-like modifier-activating enzyme 5	0.714	2.3226	Q641Q2	WASH complex subunit FAM21A	0.726	2.1581
Q9NVJ2	ADP-ribosylation factor-like protein 8B	0.713	2.7216	P31350	Ribonucleoside-diphosphate reductase subunit M2	0.686	2.4282
Q96BM9	ADP-ribosylation factor-like protein 8A	0.638	2.0669	Q8IXQ6	Poly [ADP-ribose] polymerase 9	0.59	2.0161
Q16643	Drebrin	0.635	2.1013	Q07954	Prolow-density lipoprotein receptor-related protein 1	-0.51	-4.5481
P31513	Dimethylaniline monooxygenase [N-oxide-forming] 3	0.571	2.0529	P68431	Histone H3.1	-0.511	-2.9241
Q6P179	Endoplasmic reticulum aminopeptidase 2	-0.503	-2.6892	P00505	Aspartate aminotransferase, mitochondrial	-0.519	-3.7723
P48556	26S proteasome non-ATPase regulatory subunit 8	-0.532	-2.013	Q86UP2	Kinectin	-0.522	-3.2938
Q9NVI7	ATPase family AAA domain-containing protein 3A	-0.578	-2.1811	P0C0L4	Complement C4-A	-0.526	-3.6497
Q08209	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	-0.646	-2.2221	P20073	Annexin A7	-0.531	-2.7395

Table S1: Summary of the regulated proteins by DON, ZEA and DON+ZEA after 1 h and 24 h exposure identified by LC-MS/MS.

Q9Y295	Developmentally-regulated GTP-binding protein 1	-0.65	-2.1706	Q9Y5M8	Signal recognition particle receptor subunit beta	-0.533	-2.2105
Q02880	DNA topoisomerase 2-beta	-0.691	-2.7126	Q8WWI1	LIM domain only protein 7	-0.537	-2.1471
Q5T9A4	ATPase family AAA domain-containing protein 3B	-0.741	-2.1446	Q96Q06	Perilipin-4	-0.537	-2.3202
P11387	DNA topoisomerase 1	-0.743	-3.0206	P02671	Fibrinogen alpha chain	-0.537	-3.5315
P11388	DNA topoisomerase 2-alpha	-0.752	-2.3882	P06756	Integrin alpha-V	-0.544	-2.0808
Q9UIG0	Tyrosine-protein kinase BAZ1B	-0.817	-2.7565	P84243	Histone H3.3	-0.56	-2.5582
O94919	Endonuclease domain-containing 1 protein	-0.82	-2.3091	P05026	Sodium/potassium-transporting ATPase subunit beta-1	-0.561	-2.1534
P13798	Acylamino-acid-releasing enzyme	-0.839	-2.8191	Q16775	Hydroxyacylglutathione hydrolase. mitochondrial	-0.563	-2.0028
Q9NW13	RNA-binding protein 28	-0.905	-2.4788	Q8NC51	Plasminogen activator inhibitor 1 RNA- binding protein	-0.588	-2.9397
P37059	Estradiol 17-beta-dehydrogenase 2	-0.946	-2.9482	P14678	Small nuclear ribonucleoprotein-associated proteins B and B	-0.597	-2.3388
				P11047	Laminin subunit gamma-1	-0.603	-2.308
				Q969S9	Ribosome-releasing factor 2, mitochondrial	-0.609	-2.0583
				Q92552	28S ribosomal protein S27, mitochondrial	-0.62	-2.0229
				Q86X51	Uncharacterized protein CXorf67	-0.64	-2.8233
				P02795	Metallothionein-2	-0.642	-2.6379
				P01024	Complement C3	-0.65	-4.4571
				P80297	Metallothionein-1X	-0.672	-2.9811

O60271	C-Jun-amino-terminal kinase-interacting protein 4	-0.703	-2.0434
P61026	Ras-related protein Rab-10	-0.708	-2.6567
P32321	Deoxycytidylate deaminase	-0.708	-2.1732
Q9BUJ2	Heterogeneous nuclear ribonucleoprotein U-like protein 1	-0.709	-2.8449
P45954	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	-0.712	-2.5013
Q8TD19	Serine/threonine-protein kinase Nek9	-0.718	-2.5084
P11717	Cation-independent mannose-6-phosphate receptor	-0.729	-2.6674
Q9BTZ2	Dehydrogenase/reductase SDR family member 4	-0.746	-2.1521
P42166	Lamina-associated polypeptide 2, isoform alpha	-0.771	-2.5624
Q8NFW8	N-acylneuraminate cytidylyltransferase	-0.776	-2.055
P11234	Ras-related protein Ral-B	-0.784	-2.1445
P11233	Ras-related protein Ral-A	-0.808	-2.5743
O14964	Hepatocyte growth factor-regulated tyrosine kinase substrate	-0.812	-2.009
Q96SI9	Spermatid perinuclear RNA-binding protein	-0.813	-2.3691
P62328	Thymosin beta-4	-0.816	-2.7589
A6NIZ1	Ras-related protein Rap-1b-like protein	-0.82	-3.6664
Q14165	Malectin	-0.844	-2.2966

				Q969X5	Endoplasmic reticulum-Golgi intermediate compartment protein 1	-0.848	-3.5481
				P45973	Chromobox protein homolog 5	-0.85	-2.4124
				P11387	DNA topoisomerase 1	-0.892	-2.1666
				P50402	Emerin	-0.9	-2.8165
				P62834	Ras-related protein Rap-1A	-0.916	-3.5636
				P16401	Histone H1.5	-0.969	-2.8337
				P08183	Multidrug resistance protein 1	-1.025	-3.0818
				P11388	DNA topoisomerase 2-alpha	-1.056	-2.5653
				P02751	Fibronectin	-1.088	-5.1469
				P27487	Dipeptidyl peptidase 4	-1.131	-2.8767
ZEA – 1 h				ZEA – 24 h			
ZEA – 1 h Q08431	Lactadherin	0.652	2.1646	ZEA – 24 h Q9NZ45	CDGSH iron-sulfur domain-containing protein 1	0.9	2.2476
ZEA – 1 h Q08431 P31327	Lactadherin Carbamoyl-phosphate synthase [ammonia], mitochondrial	0.652 0.61	2.1646 3.0359	ZEA – 24 h Q9NZ45 Q93034	CDGSH iron-sulfur domain-containing protein 1 Cullin-5	0.9 0.878	2.2476 2.1086
ZEA – 1 h Q08431 P31327 Q9HC38	Lactadherin Carbamoyl-phosphate synthase [ammonia], mitochondrial Glyoxalase domain-containing protein 4	0.652 0.61 0.508	2.1646 3.0359 2.0227	ZEA – 24 h Q9NZ45 Q93034 O95182	CDGSH iron-sulfur domain-containing protein 1 Cullin-5 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	0.9 0.878 0.876	2.2476 2.1086 2.5244
ZEA – 1 h Q08431 P31327 Q9HC38 P15924	Lactadherin Carbamoyl-phosphate synthase [ammonia], mitochondrial Glyoxalase domain-containing protein 4 Desmoplakin	0.652 0.61 0.508 -0.694	2.1646 3.0359 2.0227 -2.6701	ZEA – 24 h Q9NZ45 Q93034 O95182 Q9UI09	CDGSH iron-sulfur domain-containing protein 1 Cullin-5 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	0.9 0.878 0.876 0.875	2.2476 2.1086 2.5244 2.8393
ZEA – 1 h Q08431 P31327 Q9HC38 P15924 P45973	Lactadherin Carbamoyl-phosphate synthase [ammonia], mitochondrial Glyoxalase domain-containing protein 4 Desmoplakin Chromobox protein homolog 5	0.652 0.61 0.508 -0.694 -0.866	2.1646 3.0359 2.0227 -2.6701 -2.5373	ZEA – 24 h Q9NZ45 Q93034 O95182 Q9UI09 P04818	CDGSH iron-sulfur domain-containing protein 1 Cullin-5 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 Thymidylate synthase	0.9 0.878 0.876 0.875 0.875	2.2476 2.1086 2.5244 2.8393 2.226

Q9NUQ6	SPATS2-like protein	0.596	2.0428
Q9H936	Mitochondrial glutamate carrier 1	0.553	2.3223
Q15631	Translin	0.512	2.2058
P35237	Serpin B6	-0.504	-3.1496
Q6NT55	Cytochrome P450 4F22	-0.509	-2.5943
P50995	Annexin A11	-0.512	-3.1933
P04179	Superoxide dismutase [Mn], mitochondrial	-0.518	-7.771
P28482	Mitogen-activated protein kinase 1	-0.522	-2.7498
P02786	Transferrin receptor protein 1	-0.529	-2.5949
Q9NUI1	Peroxisomal 2,4-dienoyl-CoA reductase	-0.531	-2.0813
P69905	Hemoglobin subunit alpha	-0.531	-2.0409
O60610	Protein diaphanous homolog 1	-0.533	-2.3832
Q92878	DNA repair protein RAD50	-0.536	-2.83
Q6P179	Endoplasmic reticulum aminopeptidase 2	-0.541	-2.2892
O75340	Programmed cell death protein 6	-0.55	-2.0806
P02795	Metallothionein-2	-0.553	-2.2335
Q16772	Glutathione S-transferase A3	-0.566	-6.4081
Q13576	Ras GTPase-activating-like protein IQGAP2	-0.567	-2.1559
P29401	Transketolase	-0.567	-11.7612
Q5T4S7	E3 ubiquitin-protein ligase UBR4	-0.572	-2.3777

Q7L576	Cytoplasmic FMR1-interacting protein 1	-0.58	-2.7046
075165	DnaJ homolog subfamily C member 13	-0.58	-2.3785
P42285	Superkiller viralicidic activity 2-like 2	-0.58	-2.2649
Q969X5	Endoplasmic reticulum-Golgi intermediate compartment protein 1	-0.582	-2.6747
P21695	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	-0.598	-2.4374
Q9NRW3	DNA dC-)dU-editing enzyme APOBEC- 3C	-0.601	-2.4087
P57740	Nuclear pore complex protein Nup107	-0.61	-2.1652
Q8N5N7	39S ribosomal protein L50, mitochondrial	-0.614	-2.0006
Q92769	Histone deacetylase 2	-0.618	-2.2233
P04732	Metallothionein-1E	-0.624	-2.3502
Q86UP2	Kinectin	-0.63	-3.8146
Q8WWI1	LIM domain only protein 7	-0.636	-2.4778
Q99459	Cell division cycle 5-like protein	-0.636	-2.1557
Q96A33	Coiled-coil domain-containing protein 47	-0.648	-2.0261
Q13724	Mannosyl-oligosaccharide glucosidase	-0.659	-2.8472
Q6NUQ4	Transmembrane protein 214	-0.692	-2.0559
Q96FW1	Ubiquitin thioesterase OTUB1	-0.694	-3.2651
P05114	Non-histone chromosomal protein HMG- 14	-0.707	-2.1984
Q15121	Astrocytic phosphoprotein PEA-15	-0.718	-2.1008

P00390	Glutathione reductase, mitochondrial	-0.722	-5.7465
P28062	Proteasome subunit beta type-8	-0.725	-2.2744
Q03252	Lamin-B2	-0.739	-3.1591
O14936	Peripheral plasma membrane protein CASK	-0.744	-3.448
O15042	U2 snRNP-associated SURP motif- containing protein	-0.747	-2.4329
O60271	C-Jun-amino-terminal kinase-interacting protein 4	-0.75	-2.2153
O00170	AH receptor-interacting protein	-0.75	-2.5439
P48059	LIM and senescent cell antigen-like- containing domain protein 1	-0.761	-2.671
Q9NR28	Diablo homolog, mitochondrial	-0.765	-2.6006
O94905	Erlin-2	-0.765	-3.2764
P08183	Multidrug resistance protein 1	-0.774	-2.4784
Q13243	Serine/arginine-rich splicing factor 5	-0.792	-2.0753
Q96DI7	U5 small nuclear ribonucleoprotein 40 kDa protein	-0.793	-2.0389
P20073	Annexin A7	-0.8	-3.5692
P45973	Chromobox protein homolog 5	-0.807	-2.2464
Q96RP9	Elongation factor G, mitochondrial	-0.808	-2.487
Q8NFH4	Nucleoporin Nup37	-0.825	-2.6564
Q8NE71	ATP-binding cassette sub-family F member 1	-0.836	-2.4604

000422	Histone deacetylase complex subunit SAP18	-0.838	-2.1567
Q00059	Transcription factor A, mitochondrial	-0.843	-2.5605
P53582	Methionine aminopeptidase 1	-0.846	-2.5532
P21283	V-type proton ATPase subunit C 1	-0.847	-2.9585
P06756	Integrin alpha-V	-0.849	-2.9343
Q9BUP3	Oxidoreductase HTATIP2	-0.858	-2.4078
O95169	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	-0.862	-2.3292
P11387	DNA topoisomerase 1	-0.866	-2.1691
Q9UHG3	Prenylcysteine oxidase 1	-0.892	-3.5318
O95218	Zinc finger Ran-binding domain- containing protein 2	-0.907	-2.5301
075347	Tubulin-specific chaperone A	-0.913	-2.7169
Q03701	CCAAT/enhancer-binding protein zeta	-0.917	-2.307
O43847	Nardilysin	-0.931	-2.4081
P52888	Thimet oligopeptidase	-0.948	-2.5052
P51153	Ras-related protein Rab-13	-0.962	-2.8709
P32321	Deoxycytidylate deaminase	-0.965	-2.618
Q96FJ2	Dynein light chain 2, cytoplasmic	-1.004	-2.526
P23434	Glycine cleavage system H protein, mitochondrial	-1.01	-2.7255
P82933	28S ribosomal protein S9, mitochondrial	-1.013	-2.9805

				P11388	DNA topoisomerase 2-alpha	-1.014	-2.4943
				P62834	Ras-related protein Rap-1A	-1.015	-3.6631
				P27361	Mitogen-activated protein kinase 3	-1.058	-2.9475
				P11234	Ras-related protein Ral-B	-1.071	-2.7177
				P27487	Dipeptidyl peptidase 4	-1.079	-2.7567
				Q9Y6C9	Mitochondrial carrier homolog 2	-1.093	-5.3231
				P14678	Small nuclear ribonucleoprotein-associated proteins B and B	-1.098	-3.5313
				P37235	Hippocalcin-like protein 1	-1.126	-3.0693
				A6NIZ1	Ras-related protein Rap-1b-like protein	-1.133	-4.3347
				Q15418	Ribosomal protein S6 kinase alpha-1	-1.168	-3.0661
				P46063	ATP-dependent DNA helicase Q1	-1.198	-4.144
				015382	Branched-chain-amino-acid aminotransferase, mitochondrial	-1.236	-3.196
				P11233	Ras-related protein Ral-A	-1.289	-3.5055
				P00505	Aspartate aminotransferase, mitochondrial	-1.563	-7.7047
DON+ZEA – 1 h		-	-	DON+ZEA – 24 h	-	_	-
Q53FA7	Quinone oxidoreductase PIG3	0.877	2.3814	P31350	Ribonucleoside-diphosphate reductase subunit M2	0.967	3.5679
P21695	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	0.806	2.6737	P23921	Ribonucleoside-diphosphate reductase large subunit	0.922	3.4939
Q99447	Ethanolamine-phosphate	0.618	2.5182	Q99598	Translin-associated protein X	0.898	2.1645

	cytidylyltransferase						
075439	Mitochondrial-processing peptidase subunit beta	0.606	2.3247	Q15813	Tubulin-specific chaperone E	0.647	2.2011
P31327	Carbamoyl-phosphate synthase [ammonia], mitochondrial	0.56	2.7977	P62805	Histone H4	-0.511	-4.1331
Q9HC38	Glyoxalase domain-containing protein 4	0.554	2.1551	P11717	Cation-independent mannose-6-phosphate receptor	-0.539	-2.1781
Q9NR30	Nucleolar RNA helicase 2	-0.501	-3.7495	P01009	Alpha-1-antitrypsin	-0.562	-3.3868
Q8N766	ER membrane protein complex subunit 1	-0.521	-2.5239	Q6NT55	Cytochrome P450 4F22	-0.565	-2.865
Q29RF7	Sister chromatid cohesion protein PDS5 homolog A	-0.525	-2.1734	Q9HBI6	Phylloquinone omega-hydroxylase CYP4F11	-0.574	-3.7183
O94776	Metastasis-associated protein MTA2	-0.537	-2.1153	P62834	Ras-related protein Rap-1A	-0.575	-2.7173
Q5SSJ5	Heterochromatin protein 1-binding protein 3	-0.572	-2.4914	Q53GQ0	Very-long-chain 3-oxoacyl-CoA reductase	-0.588	-3.0665
O94826	Mitochondrial import receptor subunit TOM70	-0.592	-3.1238	Q86UP2	Kinectin	-0.588	-3.5278
Q9HD20	Manganese-transporting ATPase 13A1	-0.596	-2.3752	P48449	Lanosterol synthase	-0.626	-2.2063
Q53T59	HCLS1-binding protein 3	-0.604	-2.027	Q96989	Ribosome-releasing factor 2, mitochondrial	-0.636	-2.0581
Q14008	Cytoskeleton-associated protein 5	-0.611	-3.3763	Q8N5N7	39S ribosomal protein L50, mitochondrial	-0.638	-2.0845
Q8NE71	ATP-binding cassette sub-family F member 1	-0.617	-2.985	P06756	Integrin alpha-V	-0.643	-2.3894
Q14573	Inositol 1,4,5-trisphosphate receptor type 3	-0.66	-2.4621	Q15418	Ribosomal protein S6 kinase alpha-1	-0.649	-2.0716
Q9Y2L1	Exosome complex exonuclease RRP44	-0.665	-2.3093	P53007	Tricarboxylate transport protein, mitochondrial	-0.649	-2.8354
Q14839	Chromodomain-helicase-DNA-binding protein 4	-0.67	-3.4326	Q13576	Ras GTPase-activating-like protein IQGAP2	-0.652	-2.3255
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P30876	DNA-directed RNA polymerase II subunit RPB2	-0.715	-2.052	P32321	Deoxycytidylate deaminase	-0.656	-2.0278
Q9UH99	SUN domain-containing protein 2	-0.718	-3.1583	Q9BUJ2	Heterogeneous nuclear ribonucleoprotein U-like protein 1	-0.661	-2.7346
Q9HCU5	Prolactin regulatory element-binding protein	-0.738	-2.0284	P38435	Vitamin K-dependent gamma-carboxylase	-0.679	-2.8661
Q13724	Mannosyl-oligosaccharide glucosidase	-0.743	-2.3282	Q8NE71	ATP-binding cassette sub-family F member 1	-0.688	-2.1759
P11387	DNA topoisomerase 1	-0.752	-3.269	Q07954	Prolow-density lipoprotein receptor-related protein 1	-0.689	-5.8847
Q01831	DNA repair protein complementing XP-C cells	-0.796	-2.2816	P16401	Histone H1.5	-0.691	-2.3065
Q9BZE4	Nucleolar GTP-binding protein 1	-0.797	-2.2934	P42166	Lamina-associated polypeptide 2, isoform alpha	-0.697	-2.3373
Q14690	Protein RRP5 homolog	-0.85	-2.6095	P08240	Signal recognition particle receptor subunit alpha	-0.705	-2.9895
P46013	Antigen KI-67	-0.887	-4.349	P51572	B-cell receptor-associated protein 31	-0.707	-2.2534
Q9BVK6	Transmembrane emp24 domain-containing protein 9	-0.91	-2.403	Q15031	Probable leucinetRNA ligase, mitochondrial	-0.723	-2.1698
Q9UIG0	Tyrosine-protein kinase BAZ1B	-0.922	-3.0041	P01024	Complement C3	-0.724	-4.7939
O60264	SWI/SNF-related matrix-associated actin- dependent regulator of chromatin subfamily A member 5	-0.924	-3.3301	P53582	Methionine aminopeptidase 1	-0.727	-2.37
Q9NTI5	Sister chromatid cohesion protein PDS5 homolog B	-0.987	-2.6755	P50402	Emerin	-0.742	-2.5682

Q9NW13	RNA-binding protein 28	-1.062	-2.8907	P57088	Transmembrane protein 33	-0.758	-2.069
Q02880	DNA topoisomerase 2-beta	-1.092	-3.8303	Q15363	Transmembrane emp24 domain-containing protein 2	-0.77	-2.7311
P11388	DNA topoisomerase 2-alpha	-1.137	-3.3171	P0C0L4	Complement C4-A	-0.783	-5.1389
				P02751	Fibronectin	-0.809	-4.4525
				Q9H3N1	Thioredoxin-related transmembrane protein 1	-0.819	-2.1575
				P11387	DNA topoisomerase 1	-0.938	-2.3121
				P11388	DNA topoisomerase 2-alpha	-0.983	-2.4853

¹Positive values represent upregulation after mycotoxin treatment and negative values represent downregulation after mycotoxin treatment Bold characters correspond to common significantly regulated proteins after 1 h and 24 h exposure for a same mycotoxin **Table S2:** Main biological processes involved in HepaRG cells following 1 h and 24 hexposure of DON, ZEA and DON+ZEA; the biological processes were issued by the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) from proteins with a significantly different abundance from control

Biological process annotation	Count	%	Pvalue	Gene list
DON – 1 h	-	-	-	
Chromosome segregation	5	23.81	0.003	Q02880, Q96BM9, Q9NVJ2, P11388, P11387
Cell division	5	23.81	0.012	Q96BM9, Q9NVJ2, P11388, P11387, Q5T9A4
Protein modification by small protein conjugation	5	23.81	0.042	P48556, Q02880, Q9GZZ9, P11388, P11387
DNA topological change	3	14.29	3.39E-4	Q02880, P11388, P11387
Protein sumoylation	3	14.29	0.0431	Q02880, P11388, P11387
Embryonic cleavage	2	9.52	0.024	P11388, P11387
Resolution of meiotic recombination intermediates	2	9.52	0.024	Q02880, P11388
Meiotic chromosome separation	2	9.52	0.024	Q02880, P11388
DNA unwinding involved in DNA replication	2	9.52	0.047	Q02880, P11388
DON – 24 h				
Vesicle-mediated transport	17	30.91	7.8E-4	Q969X5, P02671, P02751, O00186, P61026, P11717, O60271, O14964, Q641Q2, Q07954, P11233, P11234, P06756, P62328, P01024, P0C0L4, P62834
Cell adhesion	14	25.45	0.011	P02671, O00186, P02751, Q8WWI1, P61026, Q86UP2, P06756, P27487, P68431, P42166, P11047, P50402, P05026, Q8NC51
Biological adhesion	14	25.45	0.011	P02671, O00186, P02751, Q8WWI1, P61026, Q86UP2, P06756, P27487, P68431, P42166, P11047,

				P50402, P05026, Q8NC51
Regulation of transport	13	23.64	0.022	P02671, P02751, O00186, Q07954, P11233, P11234, P06756, P50402, P01024, P0C0L4, O14964, P05026, P62834
Cell proliferation	11	20.0	0.035	P84243, P02751, Q07954, P06756, P27487, P08183, P61026, P11047, P50402, P20073, O14964
Cell-cell adhesion	11	20.0	0.036	P02671, O00186, P06756, P27487, Q8WWI1, P61026, P68431, P42166, P50402, Q86UP2, Q8NC51
Cell development	11	20.0	0.043	P84243, P02671, P02751, Q07954, Q96SI9, P06756, P61026, P80297, O60271, P11047, P62834
Regulation of vesicle-mediated transport	9	16.36	6.67E-4	P02671, O00186, P11233, P11234, P06756, P01024, P0C0L4, O14964, P62834
Positive regulation of transport	9	16.36	0.028	P02671, Q07954, P06756, P50402, P01024, P0C0L4, O14964, P62834, P05026
Locomotion	9	16.36	0.050	P02751, Q8IXQ6, Q07954, P11233, P06756, P27487, O60271, P11047, P05026
Exocytosis	8	14.55	0.003	P02671, P02751, O00186, P11233, P11234, P62328, O14964, P62834
Secretion by cell	8	14.55	0.047	P02671, P02751, O00186, P11233, P11234, P62328, O14964, P62834
Regulation of secretion by cell	7	12.73	0.013	P02671, P02751, O00186, P11233, P11234, O14964, P62834
Regulation of secretion	7	12.73	0.018	P02671, P02751, O00186, P11233, P11234, O14964, P62834
Single organism cell adhesion	7	12.73	0.033	P02671, P02751, O00186, P06756, P27487, Q8WWI1, P11047
Regulation of exocytosis	6	10.91	6.56E-4	P02671, O00186, P11233, P11234, O14964, P62834
Extracellular matrix organization	6	10.91	0.002	P02671, P02751, Q07954, P06756, P27487, P11047
Extracellular structure organization	6	10.91	0.002	P02671, P02751, Q07954, P06756, P27487, P11047
Hemostasis	6	10.91	0.016	P84243, P02671, O00186, P45973, P68431, P20073
Regulation of body fluid levels	6	10.91	0.029	P84243, P02671, O00186, P45973, P68431, P20073
Small GTPase mediated signal	6	10.91	0.031	Q9Y5M8, P11233, P11234, P61026, P62834, A6NIZ1

transduction				
Spermatogenesis	5	9.09	0.015	P84243, Q96SI9, P11717, Q99598, O60271
Male gamete generation	5	9.09	0.015	P84243, Q96SI9, P11717, Q99598, O60271
Phagocytosis	5	9.09	0.020	Q07954, P06756, P01024, P0C0L4, P62834
Regulated exocytosis	5	9.09	0.023	P02671, P02751, O00186, P62328, P62834
Blood vessel morphogenesis	5	9.09	0.028	P02751, Q07954, P06756, P01024, P62834
Lipid localization	5	9.09	0.031	Q07954, P06756, P08183, P00505, P01024
DNA conformation change	5	9.09	0.032	P84243, P11388, P16401, P11387, P68431
Gamete generation	5	9.09	0.033	P84243, Q96SI9, P11717, Q99598, O60271
Blood vessel development	5	9.09	0.049	P02751, Q07954, P06756, P01024, P62834
Apoptotic cell clearance	4	7.23	5.87E-4	Q07954, P06756, P01024, P0C0L4
Positive regulation of phagocytosis	4	7.23	7.73E-4	P06756, P01024, P0C0L4, P62834
Regulation of phagocytosis	4	7.23	0.002	P06756, P01024, P0C0L4, P62834
Substrate adhesion-dependent cell spreading	4	7.23	0.014	P02671, P02751, P06756, P11047
Positive regulation of endocytosis	4	7.23	0.016	P06756, P01024, P0C0L4, P62834
DNA packaging	4	7.23	0.031	P84243, P11388, P16401, P68431
Regulation of apoptotic cell clearance	3	5.43	0.002	P06756, P01024, P0C0L4
Regulation of protein activation cascade	3	5.45	0.013	P02671, P01024, P0C0L4
Endoderm development	3	5.45	0.015	P02751, P06756, P11047

Protein activation cascade	3	5.45	0.033	P02671, P01024, P0C0L4
Extracellular matrix disassembly	3	5.45	0.033	P02751, P27487, P11047
Cellular response to drug	3	5.45	0.044	P02795, P62834, A6NIZ1
Cellular response to erythropoietin	2	3.64	0.032	P80297, P02795
Response to erythropoietin	2	3.64	0.032	P80297, P02795
Positive regulation of apoptotic cell clearance	2	3.64	0.047	P01024, P0C0L4
Negative regulation of calcium ion- dependent exocytosis	2	3.64	0.047	O00186, P62834
Negative regulation of regulated secretory pathway	2	3.64	0.047	O00186, P62834
ZEA – 24 h				
Positive regulation of metabolic				P21695, O92769, P11388, O60271, O15121, O43847, P28062, P11234, O15257, P28482, O13243,
process	26	27.08	0.0395	Q9NR28, P62834, Q8NE71, O94905, Q92878, P48059, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179
Positive regulation of cellular metabolic process	26 25	27.08 26.04	0.0395 0.0255	Q9NR28, P62834, Q8NE71, O94905, Q92878, P48059, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 P21695, Q92769, P11388, O60271, Q15121, O43847, P28062, P11234, Q15257, P28482, Q13243, Q9NR28, P62834, Q8NE71, O94905, Q92878, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179
Positive regulation of cellular metabolic process Cellular response to stress	26 25 22	27.08 26.04 22.92	0.0395 0.0255 0.0363	 Q9NR28, P62834, Q8NE71, O94905, Q92878, P48059, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 P21695, Q92769, P11388, O60271, Q15121, O43847, P28062, P11234, Q15257, P28482, Q13243, Q9NR28, P62834, Q8NE71, O94905, Q92878, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 O94905, Q92878, Q92769, P11388, Q96A33, Q96FW1, O60271, P27361, Q8NFH4, O75340, P46063, Q15121, P35237, Q15418, Q99459, P05114, P11234, P04179, P28482, P57740, Q9NR28, P82933
Positive regulation of cellular metabolic process Cellular response to stress Cell proliferation	26 25 22 16	27.08 26.04 22.92 16.67	0.0395 0.0255 0.0363 0.044	 Q9NR28, P62834, Q8NE71, O94905, Q92878, P48059, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 P21695, Q92769, P11388, O60271, Q15121, O43847, P28062, P11234, Q15257, P28482, Q13243, Q9NR28, P62834, Q8NE71, O94905, Q92878, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 O94905, Q92878, Q92769, P11388, Q96A33, Q96FW1, O60271, P27361, Q8NFH4, O75340, P46063, Q15121, P35237, Q15418, Q99459, P05114, P11234, P04179, P28482, P57740, Q9NR28, P82933 P02786, Q92769, P48059, O14936, O75340, O43847, Q93034, Q15418, P05114, P06756, P27487, P08183, P04179, P04818, P28482, P20073
Positive regulation of cellular metabolic process Cellular response to stress Cell proliferation Response to nitrogen compound	26 25 22 16 14	27.08 26.04 22.92 16.67 14.58	0.0395 0.0255 0.0363 0.044 0.0127	 Q9NR28, P62834, Q8NE71, O94905, Q92878, P48059, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 P21695, Q92769, P11388, O60271, Q15121, O43847, P28062, P11234, Q15257, P28482, Q13243, Q9NR28, P62834, Q8NE71, O94905, Q92878, P02786, Q03701, Q00059, P27361, O14936, O75340, Q15418, P05114, P04179 O94905, Q92878, Q92769, P11388, Q96A33, Q96FW1, O60271, P27361, Q8NFH4, O75340, P46063, Q15121, P35237, Q15418, Q99459, P05114, P11234, P04179, P28482, P57740, Q9NR28, P82933 P02786, Q92769, P48059, O14936, O75340, O43847, Q93034, Q15418, P05114, P06756, P27487, P08183, P04179, P04818, P28482, P20073 O60610, P21695, Q92769, P51153, P27361, Q15121, P21283, A6NIZ1, Q99459, P11234, P04818, Q13243, P28482, P62834

Q99459, P05114, P04818, P28482

Response to organonitrogen compound	13	13.54	0.012	O60610, P21695, Q92769, P51153, P27361, Q15121, P21283, A6NIZ1, Q99459, P04818, Q13243, P28482, P62834
Cellular response to nitrogen compound	12	12.5	0.001	Q99459, P11234, O60610, P21695, Q92769, P51153, P28482, Q13243, P27361, P21283, P62834, A6NIZ1
Response to organic cyclic compound	12	12.5	0.042	Q99459, P11234, O60610, P21695, Q92769, P04818, P28482, P27361, Q15121, P20073, P62834, A6NIZ1
Cellular response to organonitrogen compound	11	11.46	0.001	Q99459, O60610, P21695, Q92769, P51153, P28482, Q13243, P27361, P21283, P62834, A6NIZ1
Positive regulation of phosphorus metabolic process	10	10.42	0.048	P11234, P21695, Q92878, Q92769, Q15257, P28482, P27361, O60271, Q15121, P62834
Positive regulation of phosphate metabolic process	10	10.42	0.048	P11234, P21695, Q92878, Q92769, Q15257, P28482, P27361, O60271, Q15121, P62834
Response to drug	9	9.38	0.016	P02786, Q92769, P08183, P04179, P11387, P04818, P02795, P62834, A6NIZ1
Cellular response to peptide hormone stimulus	7	7.29	0.026	Q99459, P51153, P28482, Q13243, P27361, P21283, A6NIZ1
Cellular response to peptide	7	7.29	0.027	Q99459, P51153, P28482, Q13243, P27361, P21283, A6NIZ1
Gland development	7	7.29	0.034	Q15418, P04179, P04818, P28482, Q13243, P27361, P62834
Positive regulation of kinase activity	7	7.29	0.050	P11234, Q92878, P28482, P27361, O60271, Q15121, P62834
Phagocytosis	6	6.25	0.037	P06756, P50995, Q7L576, P28482, P27361, P62834
Hepaticobiliary system development	5	5.21	0.028	Q15418, P04179, P04818, Q13243, P62834
Liver development	5	5.21	0.028	Q15418, P04179, P04818, Q13243, P62834
Activation of protein kinase activity	5	5.21	0.049	P28482, P27361, O60271, Q15121, P62834
Positive regulation of protein	5	5.21	0.049	P11234, P28482, P27361, O60271, Q15121

serine/threonine kinase activity				
Mesenchymal cell development	4	4.17	0.015	P48059, Q92769, P28482, P27361
Sensory organ morphogenesis	4	4.17	0.018	P05114, Q92769, P28482, P27361
Mesenchymal cell differentiation	4	4.17	0.018	P48059, Q92769, P28482, P27361
Cellular response to drug	4	4.17	0.020	P02786, P02795, P62834, A6NIZ1
Activation of MAPK activity	4	4.17	0.029	P28482, P27361, O60271, Q15121
Mesenchyme development	4	4.17	0.040	P48059, Q92769, P28482, P27361
Regulation of telomere capping	3	3.13	0.020	Q92878, P28482, P27361
Telomere capping	3	3.13	0.037	Q92878, P28482, P27361
DON+ZEA – 1 h	-	-	-	
Chromosome organization	13	37.14	5.0E-5	Q02880, Q9NTI5, Q9UIG0, P11388, P11387, Q14839, Q29RF7, P46013, O94776, Q14008, Q01831, O60264, Q5SSJ5
DNA metabolic process	12	34.28	7.35E-5	P30876, Q02880, Q9NTI5, Q9UIG0, P11388, P11387, P46013, O94776, Q29RF7, Q01831, O60264, Q9BZE4
Cell cycle	10	28.57	0.050	Q02880, Q9NTI5, P11388, P46013, Q14839, Q29RF7, Q9UH99, Q14008, Q01831, Q9BZE4
Mitotic cell cycle	8	22.86	0.044	Q02880, Q9NTI5, P11388, P46013, Q29RF7, Q9UH99, Q14008, Q01831
Cellular response to DNA damage stimulus	7	20.0	0.026	P30876, Q9NTI5, Q9UIG0, P11388, Q29RF7, Q01831, O60264
DNA conformation change	7	20.0	2.0E-4	Q02880, P11388, P11387, Q14839, Q01831, O60264, Q5SSJ5
Nuclear division	6	17.14	0.014	Q02880, Q9NTI5, P11388, P46013, Q29RF7, Q14008
DNA repair	6	17.14	0.020	P30876, Q9NTI5, Q9UIG0, Q29RF7, Q01831, O60264
Chromatin organization	6	17.14	0.029	Q9UIG0, P11387, O94776, Q14839, O60264, Q5SSJ5

Organelle fission617.140.019Q02880, QNT15, P11388, P46013, Q29RF7, Q14008Sister chromatid segregation514.290.006Q02880, QNT15, P11388, Q29RF7, Q14008DNA replication514.290.001Q02880, P11388, P11387, Q29RF7, Q9BZE4Chromatin remodeling514.290.001Q02880, QNT15, P11388, Q29RF7, Q14008Nuclear chromosome segregation514.290.010Q02880, QNT15, P11388, Q29RF7, Q14008Chromatin assembly or disassembly411.430.015Q9UIG0, O94776, O60264, Q5SSJ5Protein sumoylation411.430.019Q02880, P11388, P11387, Q01831DNA geometric change411.430.007Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.037Q04776, Q14839, O60264Strice regulation of gene expression, engenettic expression, engenettic expression, engenettic8.570.046Q02880, P11388, Q01831Hototic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Fendering expression, engenettic38.570.046Q02880, P11388, Q01831Embryonic cleavage25.710.040P11388, P11387Meiotic nfromosome separation25.710.040P11388, P11387	Chromosome segregation	6	17.14	0.003	Q02880, Q9NTI5, P11388, P11387, Q29RF7, Q14008
Sister chromatid segregation514.290.006Q02880, Q9NT15, P11388, Q29RF7, Q14008DNA replication514.290.001Q9UIG0, P11387, O94776, Q14839, O60264Chromatin remodeling514.290.010Q02880, Q9NT15, P11388, Q29RF7, Q14008Nuclear chromosome segregation514.290.010Q02880, Q9NT15, P11388, Q29RF7, Q14008Chromatin assembly or disassembl41.430.015QUIG0, O94776, O60264, Q5SSJ5Protein sumoylation41.430.019Q02880, P11388, P11387, Q01831DNA geometric change41.430.007Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P103Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Resolution of meiotic regression of geneence38.570.046Q02880, P11388, Q01831Embryonic cleavage25.710.040Q02880, P11388, Q01831Meiotic chromosome segration38.570.021Q02880, P11388, Q01831Embryonic cleavage25.710.040Q02880, P11388Meiotic chromosome segration25.710.040P1388, P11387	Organelle fission	6	17.14	0.019	Q02880, Q9NTI5, P11388, P46013, Q29RF7, Q14008
DNA replication514.290.008Q02880, P11388, P11387, Q29RF7, Q9BZE4Chromatin remodeling514.290.001Q9UG0, P11387, O94776, Q14339, O60264Nuclear chromosome segregation514.290.010Q02880, Q9NT15, P11388, Q29RF7, Q14008Chromatin assembly or disassembly414.330.015Q9UG0, O94776, O60264, Q5SSJ5Protein sumoylation411.430.019Q02880, P11388, P11387, Q01831DNA duplex unwinding411.430.006Q02880, P11388, Q14339, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P11387, Q01831Mitotic DNA integrity checkpoin38.570.041Q02880, P11388, Q01831Ricedition of gene38.570.041Q02880, P11388, Q01831Resolution of meiotic meiotic nuclear38.570.041Q02880, P11388, Q01831Ricedition of gene38.570.041Q02880, P11388, Q01831Resolution of meiotic meiotic38.570.041Q02880, P11388, Q01831Resolution of meiotic meiotic38.570.040Q02880, P11388, Q01831Embryonic cleavage25.710.040P11388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	Sister chromatid segregation	5	14.29	0.006	Q02880, Q9NTI5, P11388, Q29RF7, Q14008
Chromatin remodeling514.290.001Q9UIG0, P11387, O94776, Q14839, O60264Nuclear chromosome segregation514.290.010Q02880, Q9NT15, P11388, Q29RF7, Q14008Chromatin assembly or disassembl411.430.015Q9UIG0, O94776, O60264, Q5SSJ5Protein sumoylation411.430.019Q02880, P11388, P11387, Q01831DNA geometric change411.430.007Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-40Q02880, P11388, P11387, Q01831Meiotic nuclear division38.570.041Q02880, P11388, P16013Mitoic DNA integrity checkpoint38.570.037Q94776, Q14839, O60264Resolution of meiotic recombination intermediates38.570.041Q02880, P11388, Q1831Febryonic cleavage25.710.040Q02880, P11388, Q01831Meiotic chromosome separation25.710.040Q02880, P11388, Q01831	DNA replication	5	14.29	0.008	Q02880, P11388, P11387, Q29RF7, Q9BZE4
Nuclear chromosome segregation514.290.010Q02880, Q9NT15, P11388, Q29RF7, Q14008Chromatin assembly or disassembly411.430.015Q9UIG0, O94776, O60264, Q5SSJ5Protein sumoylation411.430.019Q02880, P11388, P11387, Q01831DNA geometric change411.430.007Q02880, P11388, Q14839, Q01831DNA duplex unwinding411.430.006Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-40Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P46013ATP-dependent chromatin remodeling38.570.037Q94776, Q14839, O60264Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Positive regulation of gene combination intermediates25.710.040Q02880, P11388Embryonic cleavage25.710.040P11388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	Chromatin remodeling	5	14.29	0.001	Q9UIG0, P11387, O94776, Q14839, O60264
Chromatin assembly or disassembly411.430.015Q9UIG0, 094776, 060264, Q5SSJ5Protein sumoylation411.430.019Q02880, P11388, P11387, Q01831DNA geometric change411.430.007Q02880, P11388, Q14839, Q01831DNA duplex unwinding411.430.006Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P46013ArtP-dependent chromatin emodeling38.570.046Q02880, P11388, Q01831Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Secolution of meiotic recombination intermediates38.570.046Q02880, P11388, Q01831Benbryonic cleavage25.710.040P1388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	Nuclear chromosome segregation	5	14.29	0.010	Q02880, Q9NTI5, P11388, Q29RF7, Q14008
Protein sumoylation411.430.019Q02880, P11388, P11387, Q01831DNA geometric change411.430.007Q02880, P11388, Q14839, Q01831DNA duplex unwinding411.430.006Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P46013ATP-dependent chromatin emodeling38.570.037Q04776, Q14839, Q60264Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Positive regulation of gene expression, epigenetic38.570.046Q02880, P11388, Q01831Embryonic cleavage25.710.040P11388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	Chromatin assembly or disassembly	4	11.43	0.015	Q9UIG0, O94776, O60264, Q5SSJ5
DNA geometric change411.430.007Q02880, P11388, Q14839, Q01831DNA duplex unwinding411.430.006Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P46013ATP-dependent chromatin remodeling38.570.0370.94776, Q14839, O60264Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Positive regulation of gene expression, epigenetic38.570.021Q09NR30, Q9UIG0, O60264Resolution of meiotic ecombination intermediates25.710.040P11388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	Protein sumoylation	4	11.43	0.019	Q02880, P11388, P11387, Q01831
DNA duplex unwinding411.430.006Q02880, P11388, Q14839, Q01831DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P46013ATP-dependent chromatin remodeling38.570.037094776, Q14839, O60264Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Ositive regulation of gene expression, epigenetic38.570.021Q02880, P11388, Q01831Resolution of meiotic recombination intermediates25.710.040P11388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	DNA geometric change	4	11.43	0.007	Q02880, P11388, Q14839, Q01831
DNA topological change38.579.92E-4Q02880, P11388, P11387Meiotic nuclear division38.570.041Q02880, P11388, P46013ATP-dependent chromatin remodeling38.570.037094776, Q14839, O60264Mitotic DNA integrity checkpoint38.570.046Q02880, P11388, Q01831Positive regulation of gene expression, epigenetic38.570.021Q9NR30, Q9UIG0, O60264Resolution of meiotic recombination intermediates25.710.040P11388, P11387Meiotic chromosome separation25.710.040Q02880, P11388	DNA duplex unwinding	4	11.43	0.006	Q02880, P11388, Q14839, Q01831
Meiotic nuclear division 3 8.57 0.041 Q02880, P11388, P46013 ATP-dependent chromatin remodeling 3 8.57 0.037 094776, Q14839, O60264 Mitotic DNA integrity checkpoint 3 8.57 0.046 Q02880, P11388, Q01831 Positive regulation of gene expression, epigenetic 3 8.57 0.021 Q9NR30, Q9UIG0, O60264 Resolution of meiotic recombination intermediates 2 5.71 0.040 Q02880, P11388 Meiotic chromosome separation 2 5.71 0.040 P11388, P11387	DNA topological change	3	8.57	9.92E-4	Q02880, P11388, P11387
ATP-dependent chromatin remodeling 3 8.57 0.037 094776, Q14839, O60264 Mitotic DNA integrity checkpoint 3 8.57 0.046 Q02880, P11388, Q01831 Positive regulation of gene expression, epigenetic 3 8.57 0.021 Q9NR30, Q9UIG0, O60264 Resolution of meiotic recombination intermediates 2 5.71 0.040 Q02880, P11388 Meiotic chromosome separation 2 5.71 0.040 P11388, P11387	Meiotic nuclear division	3	8.57	0.041	Q02880, P11388, P46013
Mitotic DNA integrity checkpoint 3 8.57 0.046 Q02880, P11388, Q01831 Positive regulation of gene expression, epigenetic 3 8.57 0.021 Q9NR30, Q9UIG0, O60264 Resolution of meiotic recombination intermediates 2 5.71 0.040 Q02880, P11388 Embryonic cleavage 2 5.71 0.040 P11388, P11387 Meiotic chromosome separation 2 5.71 0.040 Q02880, P11388	ATP-dependent chromatin remodeling	3	8.57	0.037	O94776, Q14839, O60264
Positive regulation of gene expression, epigenetic 3 8.57 0.021 Q9NR30, Q9UIG0, O60264 Resolution of meiotic recombination intermediates 2 5.71 0.040 Q02880, P11388 Embryonic cleavage 2 5.71 0.040 P11388, P11387 Meiotic chromosome separation 2 5.71 0.040 Q02880, P11388	Mitotic DNA integrity checkpoint	3	8.57	0.046	Q02880, P11388, Q01831
Resolution of meiotic recombination intermediates 2 5.71 0.040 Q02880, P11388 Embryonic cleavage 2 5.71 0.040 P11388, P11387 Meiotic chromosome separation 2 5.71 0.040 Q02880, P11388	Positive regulation of gene expression, epigenetic	3	8.57	0.021	Q9NR30, Q9UIG0, O60264
Embryonic cleavage 2 5.71 0.040 P11388, P11387 Meiotic chromosome separation 2 5.71 0.040 Q02880, P11388	Resolution of meiotic recombination intermediates	2	5.71	0.040	Q02880, P11388
Meiotic chromosome separation 2 5.71 0.040 Q02880, P11388	Embryonic cleavage	2	5.71	0.040	P11388, P11387
	Meiotic chromosome separation	2	5.71	0.040	Q02880, P11388

Regulation of gene expression	16	41.03	0.029	P53582, P02751, Q8NE71, P11388, P11387, P31350, Q15031, Q15418, P16401, P42166, Q15363, Q99598, P01024, Q9BUJ2, P0C0L4, P62805
Regulation of hydrolase activity	10	25.64	0.005	P02751, Q15418, Q07954, Q15363, P01009, P51572, P01024, P0C0L4, P62834, Q13576
Positive regulation of transport	7	17.95	0.048	Q07954, P06756, P51572, P50402, P01024, P0C0L4, P62834
Blood vessel development	6	15.38	0.003	P02751, Q07954, P06756, Q15363, P01024, P62834
Negative regulation of hydrolase activity	6	15.38	0.004	Q15418, Q15363, P01009, P01024, P0C0L4, Q13576
Inflammatory response	6	15.38	0.004	P02751, Q8NE71, Q9HBI6, P01009, P01024, P0C0L4
Vasculature development	6	15.38	0.0044	P02751, Q07954, P06756, Q15363, P01024, P62834
Regulation of peptidase activity	6	15.38	0.008	P02751, Q15418, P01009, P51572, P01024, P0C0L4
Cardiovascular system development	6	15.38	0.033	P02751, Q07954, P06756, Q15363, P01024, P62834
Circulatory system development	6	15.38	0.033	P02751, Q07954, P06756, Q15363, P01024, P62834
Endocytosis	6	15.38	0.049	Q07954, P06756, P11717, P01024, P0C0L4, P62834
Phagocytosis	5	12.82	0.007	Q07954, P06756, P01024, P0C0L4, P62834
G-protein coupled receptor signaling pathway	5	12.82	0.009	P53582, Q07954, P11717, P01024, Q13576
Blood vessel morphogenesis	5	12.82	0.010	P02751, Q07954, P06756, P01024, P62834
Regulation of endopeptidase activity	5	12.82	0.030	Q15418, P01009, P51572, P01024, P0C0L4
Apoptotic cell clearance	4	10.26	2.30E-4	Q07954, P06756, P01024, P0C0L4
Positive regulation of phagocytosis	4	10.26	3.040	P06756, P01024, P0C0L4, P62834
Regulation of phagocytosis	4	10.26	9.02E-4	P06756, P01024, P0C0L4, P62834
Acute inflammatory response	4	10.26	0.004	P02751, P01009, P01024, P0C0L4

Positive regulation of endocytosis	4	10.26	0.007	P06756, P01024, P0C0L4, P62834
Negative regulation of endopeptidase activity	4	10.26	0.028	Q15418, P01009, P01024, P0C0L4
Negative regulation of peptidase activity	4	10.26	0.029	Q15418, P01009, P01024, P0C0L4
Extracellular structure organization	4	10.26	0.032	P02751, Q07954, P06756, Q53GQ0
Extracellular matrix organization	4	10.26	0.032	P02751, Q07954, P06756, Q53GQ0
Regulation of endocytosis	4	10.26	0.038	P06756, P01024, P0C0L4, P62834
Regulation of apoptotic cell clearance	3	7.696	0.001	P06756, P01024, P0C0L4
Deoxyribonucleotide biosynthetic process	3	7.696	0.006	P23921, P31350, P32321
Protein heterotetramerization	3	7.696	0.010	P23921, P31350, P62805
Deoxyribonucleotide metabolic process	3	7.696	0.013	P23921, P31350, P32321
Positive regulation of apoptotic cell clearance	2	5.13	0.035	P01024, P0C0L4
Embryonic cleavage	2	5.13	0.046	P11388, P11387

Annexe III : Influence du type de plaque multi-puits utilisé sur les tests de viabilité cellulaire *in vitro* en toxicologie

Etude n°6

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Influence of multiwell plate type on cell viability assessment in *in vitro* toxicity studies

En préparation

Marie-Caroline Smith, Emmanuel Coton, Nolwenn Hymery

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1 Influence of multiwell plate type on cell viability assessment in *in vitro* toxicity studies

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11 Abstract: Cell viability is a systematically studied parameter in *in vitro* toxicity studies. In this 12 context, multiwell plates are commonly used for cell culture as they use small quantities of cells 13 and culture medium, and allow for the simultaneous study of a large number of possible assays 14 per plate compared to cell culture flasks, making it a high-throughput method. In this study, 15 results of viability assessments of human cells exposed to different mycotoxins were compared 16 between experiments performed in 96- and 24-well plates. In this framework, proliferating 17 HepaRG cells were treated during 48h with increasing doses of fusariotoxins, namely, 18 deoxynivalenol, nivalenol, T-2 toxin, fumonisin B1, zearalenone and moniliformin, individually 19 or in binary mixtures. Then, cell viability was measured using the mitochondrial tetrazolium salt 20 assay. Significant differences were observed on cell viability results depending on the microplate 21 type used. In particular, in 96-well plates, human hepatocytes appeared to be more sensitive to 22 fusariotoxins than in 24-well plates. In addition, when present together, mycotoxins showed 23 antagonism on cell viability reduction in 96-well plates whereas synergism was observed in 24-24 well plates. Thus, these findings highlight how in vitro assay setup may cause variations in 25 readout, affecting conclusions and hampering comparisons between assays.

- 26 *Keywords:* Cell viability; *In vitro*; Multiwell plates; Mycotoxins; Toxicology.
- 27 Abbreviations: DMSO: Dimethylsulfoxide; DON: Deoxynivalenol; FB1: Fumonisin B1; FUM: Fumonisins; IC:
- 28 Inhibitory concentration; MON: Moniliformin; MTS: Mitrochondrial tetrazolium salt; NIV: Nivalenol; T2: T-2
- 29 Toxin; TCT: Trichothecenes; ZEA: Zearalenone
- 30

31 **1. Introduction**

32 In *in vitro* toxicity studies, the first step to assess the toxicity of a substance of interest corresponds to 33 the measurement of cell viability after an acute exposure with the test substance. Among the large 34 range of cell viability assays, colorimetric assays, such as tetrazolium reduction, neutral red, trypan 35 blue and crystal violet assays, are very widespread. In order to carry out these tests, 96-well plates are 36 commonly used for cell culture and optical reading for data transcription. Indeed, they allow 37 performing a large number of assays per plate using a small number of cells and culture medium and 38 therefore help save time. Moreover, assay reagents may be added directly in wells prior to microplate 39 reading by spectrophotometers, which are consequently usually 96-well microplate reader. In this 40 context, these 96-well microplates can be considered as high-throughput systems compared to 48, 24, 41 12 or 6-well plates. Noteworthy, although microplates for high-throughput systems can be also be 42 found with 384, 1536 or even 3456 wells, these types are usually not used for cell viability 43 assessments, the cell number being too low to induce a sufficient coloration for optical-reading.

44 Among several drugs and toxins studied in toxicology, mycotoxins are of particular interest as they are 45 natural compounds produced by fungi that can be found on food and feed. Among these toxins, 46 fusariotoxins are of primary concern because of their high occurrence in food and feed commodities of 47 plant origin as well as their relevant toxicity for human and animal health. In particular, three 48 fusariotoxin families are particularly important because of their high toxicity and their occurrence in 49 European agricultural products: i) trichothecenes (TCT), with deoxynivalenol (DON), nivalenol (NIV) 50 and T-2 toxin (T2) being among the most occurred ones, ii) fumonisins (FUM), with fumonisin B1 51 (FB1) being the most abundantly produced member of the FUM family and iii) zearalenone (ZEA) 52 (Rodrigues and Naehrer, 2012; Smith et al., 2016). Beyond these major fusariotoxins, Fusarium genus 53 can produce other lesser-studied toxins with toxicological properties, such as MON, and called 54 emerging mycotoxins (Jestoi, 2008). However, while fusariotoxins are generally present 55 simultaneously in food and feed, their combined effects are still little studied. Yet, their possible 56 interactions can lead to antagonistic, additive or synergistic effects (Smith et al., 2016).

Alteration of liver functions is one of the most commonly described effects of acute mycotoxin exposure (Pitt, 2000). Indeed, the liver being our main detoxification organ, human hepatocytes represent one of the primary targets of these toxins, and are therefore a relevant *in vitro* standard model for xenobiotic metabolism and toxicity studies. In particular, the HepaRG human hepatoma cell line, derived from a human hepatocellular carcinoma, seems to be a surrogate for primary human hepatocytes for toxicity studies. Indeed, this cell line possesses both the primary hepatocytes metabolic performances and the growth capacity of hepatic cell lines (Guillouzo et al., 2007).

The main goal of this study was to compare the cell viability results from experiments assessed in 96-65 and 24-well plates. For this purpose, cell viability of human hepatocytes (HepaRG cells) exposed for 66 48h to different fusariotoxins (i.e. DON, MON, FB1, ZEA, NIV and T2) alone or in combination 67 (DON-MON and DON-FB1) was assessed using a MTS (3-[4,5-dimethylthiazol-2-yl]-5-(3-68 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. To our knowledge, this is the first 69 study that addresses and investigates the influence of well plate type on toxicological cell viability 70 assessments.

71 2. Materials and methods

72 2.1 **Toxins**

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73 DON standard (CAS#51481-10-8) with purity >98%, MON standard (CAS#71376-34-6) with purity 74 >98% and produced from Fusarium proliferatum, FB1 standard (CAS#116355-83-0) with purity 75 >98% and produced from Fusarium moniliforme, ZEA standard (CAS#17924-92-4) with purity >99% 76 as well as T2 standard (CAS#21259-20-1) with purity >98% and produced from Fusarium sp. were 77 purchased from Sigma-Aldrich (St. Louis, MO, USA). NIV standard (CAS#23282-20-4) with purity 78 >99% was obtained from Oskar-Tropitzsh e.K. (Marktredwitz, Germany). Standards were dissolved in 79 dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C. The highest 80 tested concentrations corresponded to the tested mycotoxin solubility limits.

81 2.2 Cell culture and conditions

82 Human hepatocytes (HepaRG) were acquired from Biopredic International (Rennes, France). They were seeded at a 2.6×10⁴ cells/cm² density and maintained in 75 cm² flasks using William's E medium 83 84 (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 85 mM L-glutamine, 1% penicillin 10 000 Units/ml and 1% streptomycin 10 000 µg/ml (Biochrom 86 GmbH, Berlin, Germany), 5 µg/ml insulin and 50 µM hydrocortisone hemisuccinate (Sigma-Aldrich) 87 at 37°C with 100% relative humidity (RH) in a 5% CO₂ atmosphere. After two weeks, HepaRG cells 88 were harvested with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Sigma Aldrich), and 89 reseeded in a fresh medium as a function of cell density, as recommended by the cell provider, before 90 cytotoxic assays. The HepaRG cells were used between passages 10 and 15.

91 2.3 Cytotoxicity assessment by MTS assay

92 The individual cytotoxic effect of DON, MON, FB1, ZEA, NIV and T2, as well as the combined 93 effect of DON-MON and DON-FB1 mixtures were explore on undifferentiated HepaRG cells. For this 94 purpose, HepaRG cells were seeded at a density of 1.5×10⁵ cells/cm² in both 96- and 24-well plates 95 and medium was replaced 2 days later (when about 100% confluence was observed) with fresh

96 medium containing mycotoxins dissolved in DMSO. Then HepaRG cells were incubated at 37°C for 97 48h, as it is the most commonly used exposure duration in mycotoxin cytotoxicity assessment and thus 98 allowed comparison with the maximum number of studies. Control cultures without mycotoxin but 99 with 2% solvent (DMSO) were used as negative controls. Therefore, for 96-well plates, about 5×10^4 100 cells were treated per well using 100 μ l of medium while for 24-well plates, 3×10^5 cells per well were 101 treated using 600 µl of medium. The growth area was about 0.32 cm² and 2 cm² for 96- and 24-well 102 plates respectively. Thus, the same ratio of cells/µl of medium/cm² was used for experiments carried 103 out in 96- and 24-well plates. In addition, both Falcon plates are flat-bottom tissue culture plates in 104 polystyrene and have been treated by vacuum gas plasma (Corning Inc., Corning, NY, USA). 105 Concerning combined exposure conditions, two mycotoxin mixtures were selected, namely: DON-106 MON and DON-FB1, and five concentration combinations were tested for each mixture. More 107 precisely, 0.2, 2, 6, 8 and 10 µM of DON were used in combination with 10 µM of either MON or 108 FB1. Dose-response curves were obtained by plotting the percentage of cell viability against the log of 109 mycotoxin concentrations. Then, the inhibitory concentrations (concentration inhibiting 30% and 50% 110 of cell viability from the negative control) were obtained from theoretical dose-response curves 111 established with Microsoft TableCurve 2D Software v.5.01 (Microsoft, Redmond, WA, USA).

Mycotoxin cytotoxicity towards HepaRG lineage cells was evaluated using Promega CellTiter 112 113 96AQ_{ueous} Non-Radioactive cell proliferation assay (Promega, Madison, WI). This colorimetric method 114 determines mitochondrial activity based on the reduction of a tetrazolium salt (MTS assay) by 115 mitochondrial dehydrogenases in viable cells. After 48h incubation in the presence of the mycotoxins 116 alone or in combination under normal incubation conditions, culture media was removed and cells 117 were washed with PBS. For cells seeded in 24-well plates, an additional step of harvesting by a short 118 time exposure (< 5 min) to 100 μ l of a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA was 119 performed. To stop EDTA-trypsin action, 400 µl of William's E medium containing 10% FBS were 120 added in each well and the resulting volume was transferred in a tube prior to centrifugation. 121 Supernatant was removed and cells were transferred in 96-well plates. Then, 100 µL PBS were added 122 in each well and supplemented with 20 µL CellTiter 96AQueous Non-Radioactive solution. The cells 123 were further incubated for 3 h. Formazan (MTS metabolite) was then quantified by reading the 124 absorbance at 490 nm on a Multiskan FC plate reader (Thermo Scientific, Madison, WI). Each 125 experiment was performed with biological triplicates.

126 2.4 Statistical analysis

For MTS assays, cell viability mean percentages of three independent experiments ± standard error of mean (SEM) were used for statistical analyses. Results obtained from MTS co-exposure experiments were analyzed following the model used by Weber et al., (2005) and others, which we previously detailed (Smith et al., 2016). This method is based on the comparison of theoretical expected values calculated on the basis of mono-exposure experiment results with actual values obtained from MTSco-exposure experiments.

133 No statistical difference between expected and measured mean values is interpreted as an additive 134 effect on cell viability reduction. If, the measured mean value is significantly below the expected 135 values, results are interpreted as a synergistic effect. On the contrary, when the measured mean value 136 is significantly above the expected values, it is associated with an antagonistic effect.

137 **3. Results and Discussion**

In this study, we focused on the cytotoxicity of 6 relevant fusariotoxins individually (namely DON, NIV, T2, FB1, ZEA and MON) and 2 mycotoxin combinations (DON-MON, and DON-FB1) on HepaRG cells after 48h. Cell viability was measured by the MTS assay, which is a commonly used colorimetric cytotoxicity assay based on the mitochondrial activity of the cell. Results obtained from experiments carried out in 96-well plates were compared to those obtained in 24-well plates.

143 The obtained results showed that, after 48h, whichever the multi-well plate used, exposure of HepaRG 144 cultures to single fusariotoxin led to cell viability reduction in a dose-dependent manner, except for 145 FB1 in experiments performed in 24-well plates and MON for both experiments (Figures 1). The 146 concentration-effect curves clearly exhibited much steeper slopes for DON, ZEA, NIV and T2 in 24-147 well plates than in 96-well plates. In addition, the curve maximum was generally lower for 96-well 148 plates than for 24-well plates. In particular, the observed cell viability reduction for assays carried out 149 in 96-well plates was predominantly higher than in 24-well plates, except for NIV and T2 at the 150 highest tested doses, where cell viability reduction seemed to be lower in 96-well plates than in 24-151 well plates. These observations were correlated with the determined IC_{30} and IC_{50} values 152 (concentrations of mycotoxins resulting in 30% and 50% inhibition of cell viability) (Table 1). Indeed, 153 significant differences were obtained for a given mycotoxin between assays performed in 96- and 24-154 well plates (P<0.05), even if most of these IC_{30} and IC_{50} values were in the same order of magnitude. 155 Moreover, all the IC values obtained from 96-well plates were lower than those obtained from 24-well 156 plates, highlighting a higher apparent cytotoxicity of these mycotoxins for assays performed in 96-well plates. Nevertheless, based on IC values, the studied fusariotoxins affected HepaRG cells growing in 157 158 both 96- and 24-well plates in the same following order: T2 > NIV > DON > ZEA. These results are in 159 accordance with data from literature indicating, for instance, that T2 was found to be a strong toxicant 160 compared to other fusariotoxins, while NIV was described as one of the most toxic type B TCT and 161 DON as one the weakest ones (Yazar and Omurtag, 2008).



Figure 1: Effect of DON (A), MON (B), FB1 (C), ZEA (D), NIV (E) and T2 (F) on HepaRG cell
viability after 48h exposure in 24-well plates (◆) or 96-well plates (◇) using MTS bioassay (mean
percentage ± SD of cell viability, N=3) *Mean measured cell viability significantly different from
negative control (100%) (P<0.05)

169 **Table 1:** Concentration (μ M) ±SD of mycotoxins resulting in 30% and 50% inhibition of cell viability

170 (IC₃₀ and IC₅₀, respectively) after 48h exposure (calculated using TableCurve 2D software)

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96-well pla	te cultures	24-well pla	-	
$IC_{30}(\mu M)$	$IC_{50}(\mu M)$	$IC_{30}(\mu M)$	$IC_{50}(\mu M)$	
DON	0.27 ± 0.2^{b}	1.52 ± 0.7^{b}	2.34 ±0.5	7.35 ± 0.6
MON	4.32 ± 2.7^{b}	>10 ^a	> 10 ^a	> 10 ^a
FB1	5.15 ± 0.3^{b}	>10ª	> 10 ^a	> 10 ^a
ZEA	4.52 ± 3.6^{b}	31.96 ± 5.0^{b}	33.14 ± 1.3	55.12 ±4.7
NIV	0.20 ± 0.1^{b}	0.97 ± 0.1^{b}	2.25 ± 0.2	2.84 ± 0.1
Τ2	0.09 ± 0.0	0.15 ± 0.0^{b}	0.07 ± 0.0	0.19 ± 0.0

^a Highest tested concentration

^b Significant difference (P<0.05) as compared with IC_{30} and IC_{50} obtained from assays performed in 24-well plates

172 Nowadays, the co-occurrence of these fusariotoxins in food and feed is well-known, and can be 173 explained by numerous reasons such as the facts that Fusarium species are able to produce several 174 mycotoxins simultaneously but also that matrices can be contaminated by different fungi species 175 concomitantly (Smith et al., 2016). However, few data are currently available concerning the health 176 risk from mycotoxin combined exposure. Thus, it is of great importance to assess the combined 177 toxicity of mycotoxins since a cocktail of mycotoxins can lead to a possible higher risk of adverse 178 effects than the intake of one mycotoxin alone. Indeed, mycotoxin interactions can lead to synergistic 179 effects but antagonistic or additive effects can also be observed. Here, we focused on the combined 180 effects of two relevant mycotoxin mixtures on HepaRG cell viability, namely DON-MON and DON-181 FB1 and evaluated again the influence of the multi-well plate type on viability results.

182 Results of combined exposures showed, by comparison to the expected values with the measured 183 values, that DON-MON and DON-FB1 mixtures seemed to induce mainly antagonism on cell viability 184 reduction for assays performed in 96-well plates (Figures 2.A and 2.C). On the contrary, synergism 185 was observed for assays carried out in 24-well plates (Figures 2.B and 2.D). Thus, although the same 186 concentrations were used for these cytotoxicity assessments, opposite combined effects on cell 187 viability reduction were obtained depending of the type of multiwell plate used. Interestingly, as for 188 mono-exposure experiments, the observed cell viability was higher for both mycotoxin mixtures for 189 assays carried out in 24-well plates compared to 96-well plates.



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Figure 2: Effect of DON combined with MON using (A) 96-well plates and (B) 24-well plates, and DON combined with FB1 using (C) 96-well plates and (D) 24-well plates on HepaRG cell viability after 48h incubation (mean percentage ± SEM of cell viability quantified using MTS bioassay, N=3); mean measured cell viability significantly above the expected cytotoxicity effect (antagonistic effect) ; + mean measured cell viability significantly lower the expected cytotoxicity effect (synergistic effect) ; = mean measured cell viability not significantly different from the expected cytotoxicity effect (additive effect) ; (P<0.05)

199 A comparison with the literature showed that no *in vitro* study was conducted on the combined effect 200 of DON-MON mixture, although they are commonly encountered together in food products (Smith et 201 al., 2016). Contrariwise, combined cytotoxic effects of DON-FB1 mixture have already been studied 202 by several authors (Ficheux et al., 2012; Kouadio et al., 2007; Wan et al., 2013). Conclusions varied 203 considerably from a study to another depending of cell model, incubation time for example. For 204 instance, Wan et al. (2013) reported antagonistic effects on intestinal porcine epithelial cells after 48h 205 exposure of 0.5 μ M of DON in mixture with 20 μ M of FB1, and synergism at higher doses (2 μ M of 206 DON with 40 μ M of FB1), while Kouadio et al. (2007) observed additivity on human epithelial 207 colorectal adenocarcinoma cells after 72h exposure with 10 µM of FB1 in combination with 4-20 µM

of DON. Noteworthy, only Wan et al. (2013) specified the used cell number per well and the type of multi-well plates (namely 2.5×10^4 cells/well in 96-well plates).

210 A recent review reported that the variation and occasionally low sensitivity of *in vitro* assays 211 predicting systemic toxicity could be partly explain by the difference in the bioavailability of test 212 chemicals between in vitro assays (and between in vitro and in vivo test systems) (Groothuis et al., 213 2015). The bioavailable and biologically effective dose refers to the fraction of test chemical in a 214 system that is available for uptake into cells or tissue. For instance, drugs may differentially and non-215 specifically bind to extracellular matrices such as serum proteins and plastic of well plates or other lab 216 equipment used in sample handling, and may also evaporate, degrade or be metabolized over the 217 exposure period at different rates. Herein, for an easier comparison of experiments performed in 96-218 and 24-well plates, we selected the same type of plates as well as the same ratio of cells/volume of 219 medium/surface area for both assays. Thus, as quantities were proportional between 96- and 24-well 220 plates, bioavailability of the tested mycotoxins was expected to be the same. Furthermore, in vitro 221 kinetics probably did not influence the analysis as suggested by the low log P (or log $K_{\rm OW}$) values of 222 the mycotoxins (i.e. -0.71, 1.84, 3.58, -2.24 and 2.27 for DON, FB1, ZEA, NIV and T2 respectively), 223 indicating that these compounds are not lipophilic and that they should rather be dissolved in the 224 medium than bound to protein, lipid or plastic. Therefore, the observed differences between assays 225 performed in 96-well plates and 24-well plates could be explained by the following reasons: i) the 226 difference in cell numbers: cells were about 6-fold less numerous in 96-well plates than in 24-well 227 plates, ii) the pipetted volumes were also 6-fold lower in case of 96-well plates and iii) the additional 228 step performed for tests performed in 24-well plates for harvesting the cells before optical reading in 229 96-well plates. Thus, the low number of cells used in assays performed in 96-well plates (i.e. 5×10^4 230 cells/well) may not be representative enough of the cell population. In addition, the very low pipetted 231 volumes in assays performed in 96-well plates, mostly regarding toxin volumes (i.e. $\leq 2 \mu$), may be a 232 source of inaccuracy. These hypotheses could be supported by the observed variations on mono-233 exposure experiment curves for 96-well plates compared to 24-well plates as well as higher SEM. 234 Interestingly, contrary to what was expected, the additional step of harvesting in case of 24-well plates 235 didn't seem to reduce the cell viability compared to assays performed in 96-well plates. Indeed, cell 236 viability seemed to be higher in experiments performed in 24-well plates than in 96-well plates at 237 similar toxin doses although the harvesting step in 24-well plates using trypsin-EDTA could lead to 238 cell stress and possibly even a cell membrane lysis.

The obtained results for mono-exposure experiments highlighted significant differences on cell viability reduction between assays performed in 96-well plates and 24-well plates, even if the IC_{30} and IC_{50} values were mostly in the same order of magnitude for each tested toxin. However, regarding combined exposure experiments, opposite effects on cell viability reduction were obtained between 243 experiments performed in 96- and 24-well plates. Indeed, while the assays on cell viability after 48h of 244 exposure performed in 96-well plates tend to show that multi-exposure to fusariotoxins in food 245 commodities may be less or equal cytotoxic than the presence of mycotoxins alone, the obtained 246 results for 24-well plates suggested the contrary. Thus, this study underlined that the number of cell 247 had an impact on the assay sensitivity: by miniaturizing the assay, its sensitivity seemed to decrease. 248 However, assays performed in 12-well plates did not show significant differences compared to 24-well 249 plates (data not shown). So, the cell number seemed to have an influence on the cell viability results 250 up to a maximal threshold. Above this threshold, the sensitivity of the test doesn't longer significantly 251 evolve. The surface of wells from 96-, 24- and 12-well plates are about 0.32 cm², 2 cm² and 3.8 cm² 252 respectively. Thus, number of cells and volumes are about 6-fold higher in 24-well plates compared to 253 96-well plates, and about 2-fold higher in 12-well plates compared to 24-well plates. This difference 254 could partly explain these observed results. In order to understand the observed differences at the 255 cellular level between 96- and 24-well plates, it would be very interesting to analyze the 256 transcriptome, proteome and/or metabolome of the cells after mycotoxin exposures. Here, from the 257 obtained data, we can only assume that the cell number might have an influence on cell physiology, 258 such as a possible reduction of cell-cell interactions when present in low quantity, which therefore 259 could probably reduce cell sensitivity to mycotoxin exposures.

260 Some authors have already evaluated the impact of endpoint (e.g. cell proliferation vs cell survival), 261 assay type (e.g. tetrazolium reduction vs neutral red assay), exposure duration and cell concentration 262 in toxicology studies (Altman et al., 1993; Clarke et al., 2014; Fotakis and Timbrell, 2006; Gülden et 263 al., 2001, 2005, 2010; Petty et al., 1995). However, only very few papers discussed the influence of 264 well plates on toxicity readouts. For instance, Riedl and Altenburger (2007) reported that, when using 265 microplate assay's one should be aware that lipophilic and volatile chemicals might be underestimated 266 in their toxicity compared to standardized algal growth inhibition test conducted in air tight glass 267 vessels. Herein, to the best of our knowledge, this is the first time that the influence of microplate type, 268 and consequently of cell number, on toxicological cell viability assessments (using the same cell 269 concentration) was studied. The present study highlighted the strong incidence of the methodological 270 approach used on cell viability, a parameter of great importance in many toxicological studies. Thus, 271 this study supports the need for method standardization for *in vitro* toxicity studies.

272 **Conflicts of interest:** The authors declare that there are no conflicts of interest.

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Incidence de la multi-contamination aux mycotoxines de *Fusarium* sur cellules humaines : évaluation de la cytotoxicité et approche toxico-protéomique

Les céréales et les produits issus de leur transformation sont susceptibles d'être contaminés par des espèces fongiques capables de produire des mycotoxines. L'Homme est ainsi exposé tout au long de sa vie à travers son alimentation à ces contaminants naturels, généralement à de faibles doses et en mélange. Cependant, l'incidence de la présence simultanée de ces toxines sur notre santé, à court terme comme à plus long terme, ainsi que les mécanismes responsables de leur toxicité sont encore peu ou mal caractérisés. L'utilisation de modèles cellulaires humains pertinents et adaptés est particulièrement importante pour de telles études. L'épithélium intestinal et le système immunitaire, qui constituent la première barrière de défense de l'hôte suite à l'ingestion de contaminants alimentaires, ainsi que le foie, de par son rôle majeur dans la biotransformation des xénobiotiques, représentent des modèles d'étude pertinents en toxicologie. Dans le cadre de cette étude, des modèles cellulaires humains d'origine intestinale (Caco-2), immunitaire (THP-1) et hépatique (HepaRG) ont été employés pour évaluer le risque associé à la co-exposition aux mycotoxines de Fusarium (appelées fusariotoxines) qui sont parmi les plus problématiques dans nos régions. Différents types d'interactions, tels que de l'antagonisme et du synergisme, ont pu être observés sur la viabilité cellulaire en fonction de la nature du mélange, des doses testées, de la lignée cellulaire étudiée et du modèle mathématique utilisé pour prédire les effets combinés. Des interactions ont également été observées à l'échelle moléculaire, les effets des mélanges étant très différents de ceux induits par les toxines individuellement sur le protéome des cellules. D'autres résultats obtenus interrogent sur la facon dont les mycotoxines déclenchent réellement la réponse cellulaire. De plus, les interactions entre cellules cocultivées semblent capables de modifier la réponse cellulaire suite à l'exposition à ces toxines. Ces résultats soulignent l'importance de développer des modèles in vitro de plus en plus sophistiqués et s'approchant des conditions in vivo pour permettre une meilleure caractérisation du risque « mycotoxine ».

Mots clés : Fusariotoxines, mélanges, toxicité aigüe, toxicité chronique, toxico-protéomique, THP-1, Caco-2, HepaRG, cocultures

Incidence of *Fusarium* mycotoxins multicontamination on human cells: cytotoxicity evaluation and toxicoproteomic approach.

Cereals and cereal-based products are susceptible to be contaminated by mycotoxin-producing fungi. Thus, through their diet, humans are exposed throughout their life to these natural food contaminants, mostly at low doses and in mixture. However, the health impact of the simultaneous exposure to these toxins, in acute and chronic conditions, as well as the mechanism related to their toxicity, are still poorly characterized. The use of relevant and suitable human cell models is therefore of particular importance for such studies. The intestinal epithelium and immune system, which constitute the first host defense barrier following the food contaminant uptake, as well as the liver, due to its major function in xenobiotic biotransformation, are relevant for toxicity studies. In the framework of study, the intestinal (Caco-2), immune (THP-1) and hepatic (HepaRG) human cell models were used for risk assessment associated with co-exposure to Fusarium mycotoxins (called fusariotoxins) which are the most problematic in our regions. Different type of interactions, such as antagonism and synergism, were observed on cell viability depending on the nature of the mixture, tested concentration, studied cell line and used mathematical model to predict the combined effects. Interactions were also highlighted at the molecular level, the effects of mixtures being very different from those induced by the toxins alone on the cell proteome. Other results raised the question about how mycotoxins actually trigger the cellular response. In addition, cell-cell interactions in co-cultured systems appeared to modify the cellular response following exposures to these toxins. Overall, the obtained results highlighted the relevance of developing *in vitro* models increasingly sophisticated and closer to *in vivo* conditions to allow for a better characterization of the "mycotoxin" risk.

Keywords: Fusariotoxins, mixtures, acute toxicity, chronic toxicity, toxico-protéomic, THP-1, Caco-2, HepaRG, co-culture