UNIVERSIDADE ESTADUAL DE LONDRINA

FABIANA FELIPIN RIGOBELLO

ANÁLISE QUANTITATIVA DE OCRATOXINA A (OTA) EM PLASMA HUMANO POR ENSAIO IMUNOENZIMÁTICO, E EFEITO CITOTÓXICO *IN VITRO* DE OTA E FB1 EM LINHAGEM CELULAR JURKAT E P3U1

Londrina
2015

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Tese apresentada ao Programa de Pós-graduação em Patologia Experimental, da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutor.

Orientadora: Prof.ª Dra. Eiko Nakagawa Itano.
Co-orientadora: Prof.ª Dra. Elisa Yoko Hirooka.

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Este trabalho foi desenvolvido no Laboratório de Imunologia Aplicada, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da Universidade Estadual de Londrina, sob a orientação da Prof.ª Drª Eiko Nakagawa Itano e contou com apoio financeiro da Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Araucária, Conselho Nacional de Desenvolvimento científico e Tecnológico (CNPQ) e PROPPG/UEL.

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RIGOBELLO, Fabiana Felipin. **Análise quantitativa de Ocratoxina A (OTA) em plasma humano por ensaio imunoenzimático, e efeito citotóxico *in vitro* de OTA e FB_1 em linhagem celular Jurkat e P3U1.** 2015. 59 f. Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

Micotoxinas são metabólitos secundários de fungos pertencentes aos gêneros *Aspergillus*, *Penicillium* e *Fusarium* e são contaminantes naturais de alimentos no Brasil. Ocratoxina A (OTA) e Fumonisina B_1 (FB_1) são classificados como possível carcinógenos para humanos e animais (grupo 2B). Esta pesquisa objetivou introduzir metodologia de ELISA para analisar e quantificar a presença de OTA em plasma humano e avaliar o efeito de OTA e FB_1 *in vitro* em linhagem celular P3U1 e Jurkat. Na primeira etapa do trabalho: (1) investigou-se a presença de OTA no plasma de pacientes por ELISA competitivo indireto usando anticorpos monoclonais (AcM) anti-OTA; (2) determinou-se a estimativa de ingesta diária de OTA por estes pacientes e; (3) avaliou-se a correlação da presença de OTA com marcadores plasmáticos de lesão hepática e renal. Os resultados obtidos demonstraram que OTA foi detectada em 55% das amostras (733,6 ± 296,04 pg/mL, máximo 1584,6 pg/mL), com uma estimativa de ingesta diária de 983,1-1445,3 pg/kg de peso corporal. Não houve correlação entre os níveis de OTA e parâmetros bioquímicos (AST, ALT, ureia e creatinina). Na segunda etapa, avaliou-se: (1) o efeito citotóxico de OTA e FB_1, individualmente ou em associações, sobre linhagens celulares P3U1 e Jurkat, através do método de brometo de [3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio] (MTT) e dosagem de lactato desidrogenase (LDH) e; (2) a interação de AcM anti-OTA em células tratadas com OTA por imunocitoquímica. A viabilidade das células foi reduzida com exposição à 90 µg/mL de micotoxinas após 24 h, sendo que OTA reduziu a viabilidade de P3U1 e Jurkat de forma semelhante, e FB_1 somente de célula Jurkat. O uso associado de OTA e FB_1 não aumentou o efeito citotóxico sobre as células. Os métodos de MTT e LDH apresentaram forte correlação nas células Jurkat (r = 0,749) e P3U1 (r = 0,931). Imunocitoquímica confirmou a diminuição da viabilidade celular de Jurkat e P3U1 tratadas com OTA, bem como aumento de grânulos intracelulares marcados com substrato peroxidase e destruição da membrana plasmática com liberação do citosol, indicando a presença de OTA nestas células. Conclui-se que pessoas no Brasil estão contaminadas por OTA, porém o nível de contaminação está abaixo do limite tolerável divulgado por órgãos internacionais, e possivelmente esse é o motivo pelo qual não se observou correlação entre os níveis plasmáticos de OTA e parâmetros bioquímicos de lesão hepática e renal. Conclui-se com os resultados *in vitro* que a presença de OTA induz citotoxicidade, mas nas condições estudadas, a associação de OTA e FB_1 não aumentou o efeito citotóxico.

Palavras-chave: Anticorpo monoclonal. Citotoxicidade. Fungo. ic-ELISA. Micotoxinas.

RIGOBELLO, Fabiana Felipin. **Quantitative analysis of Ochratoxin A (OTA) in human plasma by enzyme-linked immunosorbent assay, and *in vitro* cytotoxic effect of OTA and FB_1 on Jurkat and P3U1 cell lines.** 2015. 59 p. Thesis (Doctoral degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Mycotoxins are secondary metabolites of some fungi from the genra *Aspergillus*, *Penicillium* and *Fusarium* and are natural contaminants of several foods in Brazil. Ochratoxin A (OTA) and Fumonisin B_1 (FB_1) are classified as possible carcinogens to humans and animals (group 2B). Current research aimed to introduce the use of ELISA to analyze and quantify the presence of OTA in human plasma and to evaluate the effect of OTA and FB_1 *in vitro* on P3U1 and Jurkat cell lines. In the first phase of the research: (1) indirect competitive ELISA with monoclonal antibodies (mAb) anti-OTA was employed to detect the presence of OTA in human plasma; (2) the average daily intake of OTA was estimated and; (3) correlation was calculated between OTA and biomarkers for liver and kidney damage. Results showed that OTA was present in 55% of the samples (733.6 ± 296.04 pg/mL, maximum of 1584.6 pg/mL), with an estimated daily intake of 983.1-1445.3 pg/kg body weight. There was no correlation between the levels of OTA and biochemical parameters (AST, ALT, urea and creatinine). In the second phase of the research, it was evaluated: (1) the effect of OTA and FB_1, individually or associated, on P3U1 and Jurkat cell lines by the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl- tetrazolium bromide (MTT) and lactate dehydrogenase dosage (LDH) assays and; (2) the interaction of mAb anti-OTA to OTA treated cells by immunocytochemistry. Cell viability was reduced after 24 h exposure to 90 μg/mL of the mycotoxins, with OTA reducing viability of P3U1 and Jurkat cells in similar ways, and FB_1 only of Jurkat cells. The associated use of OTA and FB_1 did not increase the cytotoxic effect on the cells. MTT and LDH had a strong correlation for Jurkat (r = 0,749) and P3U1 cells (r = 0,931). Immunocytochemistry confirmed the decrease of viability of P3U1 and Jurkat cells treated with OTA, with an increase of intracellular granules marked with peroxidase substrate and destruction of the plasma membrane with the release of cytosol, indicating the presence of OTA in these cells. It can be concluded that people in Brazil are contaminated with OTA, however, the level of contamination is below the limits established by international agencies, and possibly this is the reason why there was no correlation between OTA and the markers for liver and kidney damage. Also, the *in vitro* results demonstrate that OTA induces cytotoxicity, but under the studied conditions, the association of OTA and FB_1 did not increase the toxic effect of the mycotoxins.

Keywords: Cytotoxicity. Fungi. ic-ELISA. Monoclonal antibody. Mycotoxins.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

°C	Graus Celsius
µg	Micrograma
µL	Microlitro
µM	Micromolar
AcM	Anticorpo monoclonal
AF	Aflatoxinas
ALT	Alanina aminotransferase
AML-12	*Alpha mouse liver hepatocytes*-12 (linhagem celular)
ANVISA	Agência Nacional de Vigilância Sanitária
AST	Aspartato aminotransferase
BEN	*Balkan Endemic Nephropathy* (nefropatia endêmica dos Balcãs)
BME-UV1	*Bovine Mammary Epithelium* (linhagem celular)
BSA	*Bovine Serum Albumin* (albumina sérica bovina)
bw	*Body weight* (peso corporal)
C6 cell	*Brain Glioma C6 cells* (linhagem celular)
Caco-2	*Human intestinal cell* (linhagem celular)
CF	*Conversion Factor* (fator de conversão)
DAB	Di-Amino Benzidina
DMSO	Di-Metil Sulfóxido
DNA	*Deoxyribonucleic Acid* (ácido desoxirribonucleico)
DON	Deoxinivalenol
EDTA	*Ethylene Diamine Tetraacetic Acid* (ácido etileno diamino tetra-acético)
EFSA	*European Food Safety Authority*
ELISA	*Enzyme Linked Immunossorbent Assay* (ensaio imunoenzimático)
FAO	*Food and Agriculture Organization* (Organização das Nações Unidas para Alimentação e Agricultura)
FB_1	Fumonisina B_1
FBS	*Fetal Bovine Serum* (soro bovino fetal)
H_2SO_4	Ácido sulfúrico
HCl	Ácido clorídrico
HEPES	*4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)*

HepG2	*Human hepatocellular liver carcinoma cell line* (linhagem celular)
HPLC	*High Performance Liquid Chromatography* (Cromatografia líquida de altaeficiência)
IARC	*International Agency for Research on Cancer* (Agência Internacional de Pesquisa sobre Câncer)
IC_{50}	‘Metade da concentração inibitória máxima
ic-ELISA	ELISA competitivo indireto
kg	Quilograma
L	Litro
LD_{50}	Dose Letal 50%
LDH	Lactato desidrogenase
LoD	*Limit of detection* (limite de detecção)
LoQ	*Limit of quantitation* (limite de quantificação)
MAb	*Monoclonal antibody* (anticorpo monoclonal)
MAPKs	*Mitogen-Activated Protein Kinases* (proteínas quinases ativadas por mitógeno)
min	Minuto
mL	Mililitro
MTT	*3-(4,5-di-methylthiazolyl-2)-2, 5-diphenyl- tetrazolium bromide* ng Nanograma
O.D.	*Optical Density* (densidade ótica)
OPD	*O-phenylenediamine* (o-fenilenodiamina)
OTA	Ocratoxina A
PBS	*Phosphate-Buffered Saline* (tampão salina fosfato) pg Picograma
PPAR	*Peroxisome Proliferator-Activated Receptor* (receptores ativados por proliferadores de peroxissoma)
rpm	Rotações por minuto
RPMI 1640	*Roswell Park Memorial Institute N. 1640* (meio de cultivo para células)
RSD	*Relative Standard Deviation* (desvio padrão relativo)
SD	*Standard Deviation* (desvio padrão)
TDI	*Tolerable Daily Intake* (ingesta diária aceitável)
UHT	*Ultra High Temperature* (ultra alta temperatura)
Vero cell	*Verda Uno Cell monkey* (linhagem celular)
WHO	*World Health Organization* (Organização Mundial de Saúde - OMS)
ZEN	Zearalenona

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1. INTRODUÇÃO

Micotoxinas são metabólitos secundários de fungos pertencentes aos gêneros *Aspergillus spp*, *Penicillium spp* e *Fusarium spp* e são toxinas naturais em alimentação humana, através da ingestão de produtos de origem vegetal e animal. Micotoxinas podem estar na cadeia alimentícia através da contaminação direta, como indireta através de gêneros alimentícios (leite, carne, ovos) obtidos de animais que consumiram alimentos contaminados. O maior problema associado a animais que ingerem alimentos contaminados com micotoxinas não são episódios agudos da doença (micotoxicoses), mas a ingestão de pequenas concentrações de micotoxinas por longos períodos, a qual pode causar uma série de distúrbios metabólicos, fisiológicos e imunológicos. As toxinas mais estudadas são Aflatoxinas (AF), deoxivalenol (DON), Fumonisina B1 (FB_1), Ocratoxina A (OTA), toxina T2 e zearalenona (ZEN) (BINDER, 2007; BRYDEN, 2012; OSWALD et al., 2005; SWEENEY; DOBSON, 1998).

1.1 Ocratoxina A (OTA)

OTA é produzida por espécies de *Aspergillus* e *Penicillium,* sendo *A. carbonarius, A. ochraceus, A. westerdijkiae* e espécies da seção *Aspergillus nigri* frequentemente presentes em uma variedade de alimentos, como cereais, café verde, cacau, frutas secas, carnes e seus derivados, resultando em exposição contínua da população humana (DUARTE; PENA; LINO, 2010).

Ocratoxina A (OTA) ou (*R*)-*N*-[(5-cloro-3,4-diidro-8-hidroxi-3-metil-1-oxo-1*H*-2-benzopiran-7-il) carbonil)-L-fenilalanina, é uma micotoxina que ocorre naturalmente, sendo solúvel em solventes orgânicos, em solução aquosa de bicarbonato de sódio e levemente solúvel em água. OTA é eficientemente absorvida pelo trato gastrointestinal, distribuído pela via hematogênica principalmente para os rins, fígado, músculo e gordura, e reabsorvido pela recirculação enterohepática. Portanto a biotransformação ou o *clearance* renal é diminuído, resultando em tempo de meia vida de OTA no organismo de aproximadamente 35 dias (RINGOT et al., 2006; STUDER-ROHR; SCHLATTER; DIETRICH, 2000).

De acordo com O'Brien e Dietrich (2005), ainda não está claro se o mecanismo tóxico predominante de OTA é de natureza genotóxica ou epigenética, tais como citotoxicidade induzida por estresse oxidativo celular ou aumento da proliferação celular, devido a um desequilíbrio entre vias de sinalização intracelular proliferativa e

antiproliferativa. No entanto, tanto a carcinogenicidade quanto a citotoxicidade de OTA tem sido relacionadas a danos celulares oxidativos mediados por radicais livres.

Estudos têm mostrado que OTA é nefrotóxica, hepatotóxica, teratogênica e imunotóxica para muitas espécies de animais, sendo agente causador de adenomas e carcinomas nos rins e fígado de camundongo e rato (BENDELE et al., 1985; BONDY; PESTKA, 2000; BOORMAN et al., 1992; CASTEGNARO et al., 1998; FUKUI et al., 1987). O rim é o principal órgão-alvo da toxicidade de OTA, e os danos celulares causados por OTA no túbulo proximal são devido à formação de espécies reativas de oxigênio, como o ânion superóxido, radical hidroxila e peróxido, o qual induz uma grande variedade de lesões nos componentes celulares (SCHAAF et al., 2002; SORRENTI et al., 2013). Com doses de 1 a 4 ppm de OTA, os rins de porcos perderam sua coloração e foi observado necrose dentro de 3 a 4 meses. Em investigações ultraestruturais dos rins expostos a 0,8 ppm OTA, observou-se um processo de condensação de material celular com desaparecimento de membranas e descamação contínua na parte inferior dos túbulos contorcidos proximais (PFOHL-LESZKOWICZ et al., 2007).

Efeitos adversos, incluindo anormalidades cardíacas e hepáticas, lesões do trato gastrointestinal, e tecido linfoide foram evidenciados (HAGELBERG; HULT; FUCHS, 1989). O fígado é um dos maiores órgãos-alvo de biotransformação de OTA, sendo que estudos recentes com altas doses de OTA (aproximadamente 280 µg/kg de peso corporal) detectaram notáveis lesões hepáticas (AYDIN et al., 2003; FERRANTE et al., 2006), embora baixas doses não tenham provocado alterações patológicas relevantes (KAMP et al., 2005; RACHED et al., 2007; PALABIYIK et al., 2012). Apesar de evidências não mostrarem lesões hepáticas com baixas doses de OTA, (QI et al., 2014) sugerem que média e alta doses de OTA exercem diferentes efeitos no fígado, e cinco diferentes vias metabólicas foram induzidas após o tratamento com OTA (biossíntese do ácido biliar primário, metabolismo de xenobióticos por citocromo P450 – diretamente associados com lesão hepática; metabolismo de arginina e prolina, metabolismo de cisteína e metionina, e via receptores de sinalização ativados por proliferador de peroxissoma (PPAR) – causam doenças metabólicas). Portanto OTA induz hepatotoxicidade precoce.

OTA tem sido relacionada à nefropatia endêmica dos Balcãs em humanos (BOŽIĆ et al., 1995; PFOHL-LESZKOWICZ, 2009, PFOHL-LESZKOWICZ et al., 2002, 2007), embora a hipótese não seja completamente demonstrada, colocando outros agentes nefrotóxicos como causa primária (GROLLMAN; JELAKOVIĆ, 2007; GROLLMAN et al., 2007). OTA induz apoptose em linhagens celulares de rim e em rins de rato, por meio da

ativação de proteínas quinases ativadas por mitógeno (MAPKs), bem como inibição de transportadores e distúrbio da homeostase celular (GEKLE et al., 2000; KAMP et al., 2005; PETRIK et al., 2003; SAUVANT; HOLZINGER; GEKLE, 2005; SCHRAMEK et al., 1997).

A imunotoxicidade induzida por OTA é relacionada à atuação em mais de um aspecto no sistema imune, que se assemelha em mais de um efeito a nível celular, inibindo a imunidade celular, humoral e inata, incluindo diminuição celular em órgãos linfoides, nível de imunoglobulinas e atividade fagocítica de monócito sanguíneo em frango (ATROSHI et al., 2000; BONDY; PESTKA, 2000; CHANG; HAMILTON, 1980). OTA também inibe a atividade de célula *natural killer* (NK), a produção de interferon em camundongo (LUSTER et al., 1987), a proliferação de linfócitos T e B in vitro e anula a produção de IL-2 e seus respectivos receptores (LEA; STEIEN; STØRMER, 1989). Quando administrado a várias espécies animais, OTA induz efeitos diversos na medula óssea e na resposta imune, incluindo linfopenia em cão (SZCZECH; CARLTON; TUITE, 1973), frango (CHANG; HUFF; HAMILTON, 1979), peru (DWIVEDI; BURNS, 1985), suíno (KROGH; ELLING, 1977; Krogh et al., 1979; SZCZECH et al., 1973;) e regressão do timo e imunossupressão em camundongo (BOORMAN et al., 1984).

Primeiramente OTA foi detectada em amostras de soro humano em 1977 na Alemanha (BAUER; GAREIS, 1987) e em 1980 na Iugoslávia (Croácia) (HULT et al., 1982). Desde então, tem sido detectada em amostras de soro humano em todo o mundo: Europa (Bulgária, Croácia, Republica Tcheca, Dinamarca, França, Alemanha, Hungria, Itália, Noruega, Polônia, Portugal, Espanha, Suíça, Suécia, Reino Unido, Iugoslávia), África (Argélia, Egito, Costa do Marfim, Marrocos, Serra Leoa, Tunísia), Ásia (Japão, Líbano, Paquistão, Turquia) e nas Américas (Argentina, Chile, Costa Rica e Canadá) (ASLAM et al., 2012; BIASUCCI et al., 2011 CORONEL et al., 2011; DI GIUSEPPE et al., 2012; HMAISSIA KHLIFA et al., 2012; LEE; RYU, 2015; MARTLBAUER et al., 2009; MUÑOZ et al., 2014; SABUNCUOGLU et al., 2015).

A presença de OTA no soro/plasma humano indica a exposição contínua e sua detecção continua sendo o método básico de monitoramento da exposição à OTA (MALIR et al., 2012; SCOTT, 2005), sendo a exposição crônica a baixas concentrações mais prejudicial que a exposição aguda decorrente de concentração elevada (PFOHL-LESZKOWICZ, 2009; PFOHL-LESZKOWICZ; MANDERVILLE, 2007).

O ensaio imunoenzimático (*Enzyme linked immunosorbent assay* - ELISA) para análise de OTA é considerado um método importante e rápido, devido à facilidade de usar, processamento de um grande número de amostras ao mesmo tempo, além de não requerer

limpeza da amostra. O método de ELISA tem sido aplicado para quantificar OTA em cereais, alimentos, tecidos de animais e soro (EL KHOURY A.; ATOUI A., 2010). Além da quantificação por ELISA, a determinação de OTA por HPLC com detector de fluorescência é amplamente empregado. Estudos tem demonstrado uma boa correlação entre os métodos de detecção de OTA por HPLC e ELISA (DOHNAL et al., 2013; MARTLBAUER et al., 2009).

Desde 2006, a *European Food Safety Authority* se preocupou em estabelecer parâmetros para a ingesta máxima de OTA. Atualmente, aceita-se a ingesta semanal de 120 ng/Kg peso corporal. Em alimentos, é permitido de 0,5 µg/kg (em alimentos de bebês e crianças) a 10 µg/kg (em café solúvel e frutas secas). No Brasil, a RDC n°7/2011 da Agência Nacional de Vigilância Sanitária (ANVISA) permite concentrações de OTA entre 2-30 µg/kg dependendo do tipo de alimento (BRASIL, 2011).

1.2 Fumonisina B1 (FB_1)

Fumonisinas são micotoxinas produzidas principalmente por *Fusarium verticillioides Sacc Nirenberg* (=*F. moniliforme* Sheldon) e *Fusarium proliferatum* (TARANU et al., 2005), sendo contaminantes de milho e outros cereais (GELDERBLOM et al., 1988). Vinte e oito análogos foram caracterizados, sendo as quatro categorias de fumonisinas A, B, C e P. O análogo B compreende as fumonisinas FB_1, FB2 e FB3 de importância toxicológica, sendo FB_1 responsável por 70 a 80% do total de fumonisinas produzidas em milho e arroz (RHEEDER; MARASAS; VISMER, 2002).

As fumonisinas são estruturalmente semelhantes aos precursores dos esfingolipídeos, em especial esfinganina e esfingosina, bloqueando a biossíntese pela inibição da ceramida sintase [esfingosina(esfinganina)-N-acetiltransferase] (TURNER; NIKIEMA; WILD, 1999). Em consequência, ocorre o bloqueio da biossíntese de esfingomielina (responsável pela formação de 18-19% da membrana plasmática no fígado), do glicolipídeo neural (camada externa da mielinização da membrana celular) e do gangliosídeo (receptor membranar para sinalização entre células), além do acúmulo de esfinganina, uma importante molécula de sinalização para enzima (MOSS, 2002).

Estudos sobre a toxicidade de FB_1 *in vivo* indicam que os principais órgãos alvo são rins e fígado. Fumonisinas são pobremente absorvidas e rapidamente eliminadas via dois ou três modelos de compartimento, e não são metabolizados em animais. Os mecanismos celulares envolvidos na toxicidade induzida por FB_1 incluem a indução do estresse oxidativo, apoptose,

citoxicidade, bem como alterações na expressão de citocinas. (STOCKMANN-JUVALA; SAVOLAINEN, 2008).

A FB_1 tem sido associada a diversas doenças, a exemplo de leucoencefalomalácia equina, edema pulmonar em suíno, carcinoma hepático em rato, além de reduzir a viabilidade e atividade fagocítica de macrófagos em galinha (COLVIN; HARRISON, 1992; HOWARD et al., 2001; LI et al., 1999; MARASAS et al., 1988). Em humano, fumonisinas em milho já foi relacionada com a ocorrência de câncer esofágico em Transkei (África do Sul), China e norte da Itália (FAO-WHO, 2001). As toxinas de *Fusarium* spp. foram classificadas pela Agência Internacional de Pesquisa do Câncer (*Internacional Agency for Research on Cancer* – IARC) como possivelmente carcinogênicas ao homem (grupo 2B) (IARC, 1993). Além disso, o consumo de FB_1 pode induzir supressão do sistema imune envolvendo imunidade inata, humoral e celular em várias espécies (BONDY; PESTKA, 2000; MARTINOVA; MERRILL Jr, 1995; QURESHI et al., 1995). Apesar do potencial tóxico, carcinogênico e indutor de imunossupressão da FB_1, muitos países ainda não estabeleceram níveis máximos tolerados em alimentos. No Brasil, estudos realizados por (ONO et al., 2000, 2001) demonstraram que 98% das amostras de milho recém-colhido (safras 1995 e 1996), de três regiões do estado do Paraná continham FB_1 em concentrações médias de 0,096 a 22,6 μg/g. No Brasil, a RDC n°7/2011 ANVISA determina concentrações de FB_1 em alimentos de 200 a 1500 μg/kg, dependendo do tipo de processamento do alimento (BRASIL, 2011).

2. OBJETIVOS

2.1 Objetivo geral

Introduzir metodologia de ELISA para analisar e quantificar a presença de Ocratoxina A (OTA) no plasma humano e avaliar o efeito de OTA e Fumonisina B_1 (FB_1) em linhagem celular P3U1 e Jurkat.

2.2 Objetivos específicos

1) Padronizar a metodologia de ELISA para análise e quantificação de OTA no plasma humano;
2) Determinar a concentração de OTA no plasma humano de pacientes de duas cidades do estado do Paraná;
3) Estimar a ingesta diária de OTA pelos pacientes;
4) Investigar a correlação entre os níveis plasmáticos de OTA com marcadores plasmáticos de lesão renal e hepática;
5) Avaliar efeito citotóxico de OTA e FB_1 em linhagens celulares P3U1 e Jurkat;
6) Avaliar efeito citotóxico da associação de OTA + FB_1 em linhagens celulares P3U1 e Jurkat;
7) Comparar a sensibilidade dos métodos de MTT e LDH na avaliação de efeito citotóxico de OTA;
8) Avaliar a interação de anticorpos anti-OTA em linhagens celulares P3U1 e Jurkat tratadas com OTA, por imunocitoquímica.

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4. ARTIGO A: Plasma levels of ochratoxin A of inhabitants of northern Paraná, Brazil

Abstract

Ochratoxin A (OTA), a mycotoxin produced by some fungi like *Aspergillus ochraceus*, *A. niger*, *A. cabonarius*, *Penicillium viridicatum*, is a natural contaminant of many foods worldwide. The intake of OTA is associated with deleterious effects to humans and animals, as nephro and hepatotoxicity. Although there are some data about food contamination, there is lack of data about human exposure to OTA in Brazil. The aim of this research was to determine the level of human exposure to OTA and, additionally, identify possible associations with liver and kidney damage markers. The OTA levels were evaluated in plasma samples from 149 individuals living in the state of Paraná, Brazil, by indirect competitive ELISA using anti-OTA. OTA levels and plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine were submitted to Pearson's correlation test. OTA was detected in 55% of the samples mean (733.6 ± 296.04 pg/mL; max. 1584.6 pg/mL), with an estimated daily intake of 683-1004 pg/kg body weight. There was no correlation between OTA plasma levels and biochemical parameters, possibly due to the low level of contamination. This is the first research concerning the contamination of humans by OTA in Brazil and we conclude that the plasma levels of the evaluated population are below the limit established by the international agencies. Nevertheless, additional longitudinal studies with greater regional coverage and at different sazonal periods are necessary.

Keywords: food, fungi, hepatotoxicity, ic-ELISA, monoclonal antibody, mycotoxins, nephrotoxicity

Introduction

Ochratoxin A (OTA) is a mycotoxin that widely contaminates staple foods and beverages (PETKOVA-BOCHAROVA; CHERNOZEMSKY; CASTEGNARO, 1988; RADIĆ et al., 1997) and is classified by the International Agency for Research on Cancer (IARC) as class 2B, which is possibly carcinogenic to human (IARC, 1993). OTA has nephro,

hepato, immunotoxicity, teratogenic and possibly neuro and genotoxic properties (EFSA, 2006). When ingested as a food contaminant, OTA binds to serum albumin after a single oral dose and due to unfavorable pharmacokinetics elimination, it remains in the bloodstream for 35 days (HAGELBERG; HULT; FUCHS, 1989; PERRY et al., 2004; PETZINGER; ZIEGLER, 2000; UCHIYAMA; SAITO, 1987).

The effect of OTA in liver and kidneys is more pronounced, as both organs are involved in detoxification and elimination of OTA from the body. This toxin is excreted through kidney tubules using organic anion transporter proteins and is also reabsorbed in all nephron segments using the same transporters or possibly by others. The reabsorption process reduces OTA excretion, leading to its accumulation in renal tissue and thus contributing to renal toxicity (DAHLMANN et al., 1998; PFOHL-LESZKOWICZ; MANDERVILLE, 2007).

OTA levels in blood are considered one of the essential indicators of human exposure to this mycotoxin. OTA was first detected in human blood samples in Germany, in 1977 (BAUER; GAREIS, 1987) and former Yugoslavia (Croatia) in 1980 (HULT et al., 1982). Since then, OTA has been detected in human blood samples worldwide (ASLAM et al., 2012; BIASUCCI et al., 2011; DI GIUSEPPE et al., 2012; HMAISSIA KHLIFA et al., 2012; MALIR et al., 2001, 2013; MARTLBAUER et al., 2009; MUÑOZ et al., 2014; SABUNCUOGLU et al., 2015; ZAIED et al., 2011).

OTA is a toxin produced by *Aspergillus ochraceus*, *A. niger*, *A. carbonarius*, *A. sulphureus*, *Penicillium viridicatum*, *P. cyclopium* and other species (EFSA, 2006). The tropical/subtropical climate provides excellent conditions for the growth of mycotoxigenic fungi (PEDROSA; DEZEN, 1991). In Brazil, OTA producing fungi were already identified on coffee grains and grapes, such as *A. auricoumus*, *A. ochraceus*, *A. ostianus*, *A. niger, A. carbonarius* and *A. westerdijkiae* (DE FATIMA REZENDE et al., 2013; PASSAMANI et al., 2012; SARTORI et al., 2014). Besides, contamination was detected in foodstuffs, like raisins (ALMEIDA et al., 2006), wines and grape juices (SHUNDO et al., 2006) and in a human milk bank in Brazil (NAVAS; SABINO; RODRIGUEZ-AMAYA, 2005). A recent research demonstrated that almost 90% of UHT cow milk in the state of Paraná (Brazil) was contaminated with an average of 19.6 ng/L of aflatoxin M_1 (AFM_1), of which 2.6% had results above 50 ng/L, the maximum limit allowed established by the European Community (SILVA et al., 2005).

Considering that the genera *Aspergillus* comprises species that produce both aflatoxins and ochratoxins, the population of state of Paraná, Brazil, could be contaminated with OTA. Current research demonstrates, for the first time, the results of a quantitative

analyses of OTA levels in human blood samples, demonstrating OTA exposure of individuals living in the north region of the state of Paraná, Brazil.

Material and Methods

Samples

A total of 149 blood samples from clinical laboratories were used: 99 from Jacarezinho and 50 from Londrina, both cities in the north of Paraná state, Brazil. Blood was drawn with EDTA vacuum tubes and plasma aliquots were stored at -20 °C until use. Current research was approved by the Internal Scientific Commission and the Research Bioethics Committee of the State University of Londrina (number 23351.2012.08). The study was explained to all participants and they signed the informed consent. People excluded from the study were alcoholics, patients with viral hepatitis, with chronic renal disease or with any known liver or kidney diseases.

Sample Extract Preparation

The sample extract preparation was performed according to Martlbauer et al. (2009) with some modifications. Plasma (750 µL) was mixed with 1 M HCl (1.25 mL) and methylene chloride (2 mL) for 5 min on a magnetic stirrer. The mixture was centrifuged (1,500 x g, 15 min). Two milliliters of the organic phase were collected and extracted. After centrifugation, organic phase was collected and the solvent evaporated (rotary evaporator). The residue was dissolved with 750 µL of phosphate buffered saline (PBS)/ethanol solution (9:1).

Production of anti-OTA monoclonal antibody (MAb)

The cell line anti-OTA used was Anti-OTA.7 MAb, cross-reactivity of 79.4 % for OTC, prepared at Science University of Tokyo, Japan (KAWAMURA et al., 1989). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% L-glutamine. The supernatant was collected and precipitated with saturated solution of ammonium sulfate $[(NH_4)_2SO_4]$. The precipitate was centrifuged (30 min, 2,500 g), redissolved in PBS and dialyzed against it. The IgG antibodies were purified by Protein A

Antibody Affinity Chromatography (Affi-Prep® protein A, cat nº 156-0005, Bio-Rad Laboratories, Hercules, CA, USA), and the protein concentration determined at 280 nm.

Indirect Competitive Enzyme Linked Immunosorbent Assay (ic-ELISA)

ELISA was performed according to Dos Santos et al. (2011) with some modifications. High-affinity 96-well polystyrene plates (Costar, Corning Inc., Corning, NY, USA) were coated with 100 µL of 160 ng/mL OTA-BSA (O3007, Sigma Chemical Co., St. Louis, MO, USA) in 0.1 M carbonate-bicarbonate buffer pH 9.6, for 18 h at 4 °C. After washing four times, the wells were blocked (PBS/skim milk) for 1 h at 25 °C. After new washes, 50 µL of OTA standard (O1877, Sigma Chemical Co., St. Louis, MO, USA) or plasma samples, plus 50 µL of anti-OTA MAb (43 ng/mL) were added to the wells for 18 h at 4 °C. Anti-mouse IgG-peroxidase labeled antibody (1031-05, SouthernBiotech, Birmingham, AL, USA) was used at 1:100 dilution (100 µL) for 1 h at 37 °C, followed by the addition of o-phenylenediamine (OPD) substrate solution. After 10 minutes, the reaction was stopped by adding 50 µL of 1 M H_2SO_4 and absorption was measured at 492 nm in a Multiskan EX Reader (Labsystems, Helsinki, Finland). Tests were perfomed in duplicate and mean absorbance results were expressed as the percentage of binding: Binding (%) = (A^+/A^-) x 100, where A^+ is the mean absorbance in the presence of each sample or standard and A^- is the mean absorbance in their absence.

Precision

Precision was demonstrated as repeatability (RSDr, intra- or within-day precision), and reproducibility (RSDR, inter- or between-day precision). Each of these precision assays were determined by analyzing three replicates of plasma samples, spiked with OTA at the levels of 100, 500 and 2000 pg/mL. Within-day repeatability was performed by triplicate determination on the same day by the same operator. Between-day repeatability was performed by repeating the same procedure on three different days. The following equations were employed: Limit of detection = LoD = Mean - 3xSD; Limit of Quantitation = LoQ = Mean - 6xSD. Mean and standard deviation were obtained from the white (OTA - BSA + anti-OTA) in the repeatability and reproducibility tests. The calibration curve was established by analyzing three replicates of OTA standard in the range 65 to 2000 pg/mL.

Blood tests

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) tests were outsourced in a clinical laboratory (ALT/GPT Liquiform and AST/GOT Liquiform, used in Labmax Plenno, Labtest Diagnóstica S.A., Lagoa Santa, MG, BR). Urea (742201 Urea Enz. Color kit 5x50 mL, Laborclin Produtos para Laboratório Ltda., Pinhais, PR, BR) and creatinine (742071 Creatinine kit 2x100 mL, Laborclin Produtos para Laboratório Ltda., Pinhais, PR, BR) were dosed using standard protocols, according to the manufacturers' instructions.

OTA daily intake

Based on plasma levels of OTA, the average daily toxin intake can be estimated from renal clearance of OTA (HAGELBERG; HULT; FUCHS, 1989; BREITHOLTZ et al., 1991; MIRAGLIA; BRERA; COLATOSTI, 1996; STUDER-ROHR; SCHLATTER; DIETRICH, 2000). Two slightly different values for the renal clearance have been reported: Hagelberg, Hult and Fuchs (1989) work was based on renal clearance from animal data (0.033 mL/min), resulting in the OTA intake demonstrated in equation 1; and Studer-Rohr, Schlatter and Dietrich (2000) elaborated equation 2 based on the renal clearance of one human volunteer who ingested tritium labelled OTA (0.048 mL/min).

Tolerable daily intake (TDI) of OTA [ng/kg body weight (bw)]:

$K_0 = 0.99 \times C_p/0.5 = 1.97 \times C_p$ (equation 1) (HULT; FUCHS, 1989)

$K_0 = 0.67 \times C_p/ 0.5 = 1.34 \times C_p$ (equation 2) (STUDER-ROHR; SCHLATTER; DIETRICH, 2000)

Where (SCOTT, 2005):

K_0 = continuous dietary intake (ng/kg bw per day);

0.99 or 0.67 is the renal clearance rate (mL/kg bw per day);

0.5 is the bioavailability (fraction of OTA taken up);

C_p = plasma concentration (ng/ml)

In this research, both equations were employed to estimate the daite OTA intake.

Data analysis

Data were analyzed using the IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and are presented as mean and standard deviation (SD). Before the analysis, the following assumptions were tested in numerical variables: homogeneity of variances using the Levene's test and the data distribution using the Shapiro-Wilk's test. The two-factor ANOVA was used, followed by the multiple comparison test with significance level adjustment proposed by Sidak. The relationship between variables was investigated using the Spearman correlation.

Results and Discussion

The use of ic-ELISA to detect OTA

The most commonly used analytical method for the determination of OTA in blood samples is high performance liquid chromatography (HPLC) with fluorescence, ultraviolet or mass spectrometric detection (PETKOVA-BOCHAROVA et al., 2003; POSTUPOLSKI; KARLOWSKI; KUBIK, 2006; ZIMMERLI; DICK, 1995). However, HPLC analyses show some disadvantages as cost, time-consuming cleanup sample, use of organic solvents and the need for trained users. On the other hand, immunotechniques, such as ELISA, require simple preparation of samples and are easy and fast, however they may have cross-reactions and matrix interference. Specific MAb have been employed to detect OTA by high sensitivity ELISA on chicken meat, wheat flour, porcine plasma, bovine serum and human plasma (KAWAMURA et al., 1989; UENO et al., 1998). Some researchers demonstrated a good correlation between HPLC and ELISA to detect OTA (MARTLBAUER et al., 2009; DOHNAL et al., 2013), and therefore, ELISA was the chosen method for this research.

After precision analysis by repeatability and reproducibility, the calibration curve was established in the range 65 to 2000 pg/mL (r = 0.9932). The limit of quantitation (LoQ) was equivalent to 371 pg/mL. In the blood sample enriched with OTA at levels of 2000, 500 and 100 pg/mL, the recoveries were about 88, 101, and 85% respectively. Martlbauer et al. (2009) experiments demonstrated that the detection limit of the EIA for OTA in serum was about 50-80 pg/mL, and recoveries of OTA spiking levels of 200 pg/mL and 400 pg/mL ranged from 81 to 160%. Using the same method for analysis of OTA in porcine serum, mean

recoveries of OTA at levels of 0.5 ng/mL and 1.0 ng/mL were found to be at 71.6% and 67.5%. Figure 1 shows the standard curve of the ic-ELISA for OTA.

OTA in human plasma samples

Of the 149 plasma samples analyzed, 81 were positive for OTA, within a range of 373 to 1584.6 pg/mL, with a mean concentration of 533 ± 328 pg/mL. Of the total, 64 samples were from males and 85 from females, with a range of 375-1489 and 373-1584.6 pg/mL, respectively (table 1). Neither sex nor age show statistical differences between volunteers in the plasma levels of OTA. Several studies in Canada (FROHLICH; MARQUARDT; OMINSKI, 1991; SCOTT et al., 1998), Lebanon (ASSAF et al., 2004), Japan (UENO et al., 1998), Norway (SKAUG, 2003), Germany (GAREIS; ROSNER; EHRHARDT, 2000), Northern Spain (JIMENEZ et al., 1998), Italy (PALLI et al., 1999) and Tunisia (GROSSO et al., 2003) found no difference concerning age in positive OTA levels in human blood. As plasma OTA levels were not significantly different between genders and the age of the population examined, the source of OTA is assumed to be common dietary foodstuffs (UENO et al., 1998).

Martlbauer et al. (2009) compiled data of blood levels of OTA of apparently healthy subjects (i.e. not obviously suffering from kidney diseases) from a total of 30 countries, with the majority of results of maximum OTA of 1,000-3,000 pg/mL, but levels of 50,000 pg/mL or higher were occasionally found in various regions. Most data have been reported for European and North African countries, and there is no available data for countries with larger population and territory diversity, like Brazil. Therefore, this is the first research with Brazilians and the current results are in agreement with previously published studies (BIASUCCI et al., 2011; CORONEL et al., 2011; HASSEN et al., 2004; LEE; RYU, 2015; MAAROUFI et al., 1995a, 1995b; MALIR et al., 2001, 2013; MARTLBAUER et al., 2009; MUÑOZ et al., 2014; OMINSKI, 1991; SABUNCUOGLU et al., 2015; UENO et al., 1998).

Plasma levels of OTA and the mycotoxin's average daily intake

Using the equations mentioned in the topic "Ota daily intake" described in the Material and Methods section, the mean OTA plasma levels was 733.66 ± 296.04 pg/mL (range 372.73 to 1,584.6 pg/ml). Using the conversion factor (CF) of 1.34 or 1.97, the mean daily intake of OTA was 983.1 ± 396.7 and 1,445.3 ± 583.2 pg/kg bw, respectively (table 1).

Estimated dietary intakes of OTA in six European countries (Germany, Italy, Norway, Spain, Sweden and UK) were calculated using CF 1.97 as 350-2,340 pg/kg bw per day from serum/plasma concentrations ranging from 180-1,190 pg/mL (SCOTT, 2005). Our results are in accordance with those from Europe. Correlations between plasma levels of OTA and specific types of food consumed have been pointing to cereal products, wine, beer and pork in Norway and Sweden (THUVANDER et al., 2001) and for certain cereal products, sausages, red grape juice, chocolate with nuts and coffee in Germany (GAREIS; ROSNER; EHRHARDT, 2000). Comparisons of blood levels of OTA and food data with nutritional behavior in Germany leaded to the conclusion that multiple sources are responsible for the dietary OTA intake, making it almost impossible to avoid this mycotoxin (GAREIS; ROSNER; EHRHARDT, 2000).

The estimated weekly OTA intake found in the current research was 1,445 pg/kg bw, almost 10 times lower than the tolerable weekly intake of 120,000 pg/kg bw established by the European Food Safety Authority (EFSA, 2006). Although the estimated intake of OTA of the individuals evaluated is lower than the established by the EFSA, researches in Brazil show that there is contamination by the mycotoxin in industrialized products, such as dark chocolate (0.39 µg/Kg) (COPETTI et al., 2012), rice (0.20-0.24 µg/Kg) (ALMEIDA et al., 2012), corn based products (64 µg/Kg) (SEKIYAMA et al., 2005), paprika (7.0 µg/Kg) (SHUNDO et al., 2009), instant coffee (0.17-6.29 ng/g) (ALMEIDA et al., 2007), frozen grape juice pulp (37-100 ng/L) and wine (34.4 ng/L)(ROSA et al., 2004), at low level.

There was no significant difference in plasma levels of OTA between the participating cities (p-value = 0.704). Although Londrina and Jacarezinho have an average of 540,000 and 40,000 inhabitants, respectively, the cities are both in the north of Paraná and the consumption of processed foods predominates, i.e. these foods are produced by industries that must comply with current laws, thereby probably reducing the contamination of products by mycotoxins. It would be interesting to have a complementary study in other regions of Brazil where most food is stocked in domestic silos. Nevertheless, the simple fact that everyone is continuously exposed to dietary OTA is of some concern, and maximum levels in food have to be seen in the light of real-life exposure (MARTLBAUER et al., 2009).

Blood tests: AST, ALT, Urea and creatinine

The liver is one of the major target organs of OTA biotransformation. Although some early hepatotoxicity studies employed relatively high concentrations of OTA and found

remarkable liver lesion (AYDIN et al. 2003; FERRANTE et al., 2006), lower doses of OTA did not provoke significant pathological changes (KAMP et al., 2005; PALABIYIK et al. 2012; RACHED et al., 2007). Current research evaluated the levels of blood markers for liver damage and correlated them with OTA concentrations in plasma (Table 1). The mean plasma levels of these markers (AST 21.1 ± 5.54 U/L; ALT 21.81 ± 7.62 U/L) are within the normal range disclosed in the test kit (AST 11-41 U/L; ALT 7-52 U/L). There was no correlation between plasma levels of OTA and the liver enzymes (AST r= -0,07010; ALT r= 0,03402).

Some researchers have demonstrated the effects on AST/ALT of different doses of OTA given to rats (KAMP et al., 2005a, 2005b; MALLY et al., 2005; GAGLIANO et al., 2006; ARBILLAGA et al., 2007, 2008), mice (FERRANTE et al., 2006), hens (DENLI et al., 2008) and chicken (ELAROUSSI et al., 2006). The blood levels of markers for liver damage increased because of OTA intake, contrary to the albumin level that decreased linearly, showing the detrimental effect of the mycotoxin on protein synthesis in the liver (DI GIUSEPPE et al., 2012). Recently, Qi et al. (2014) demonstrated that medium and high doses of OTA exert different effects on the liver. Five distinct pathways were induced after OTA treatment. Two pathways are directly associated with liver damage (primary bile acid biosynthesis and metabolism of xenobiotics by cytochrome P450), whereas the remaining pathways arginine and proline metabolism, cysteine and methionine metabolism and peroxisome proliferator-activated receptor α (PPAR) cause metabolic disease. However, all these studies employed higher doses than the estimated intake calculated for patients of the current study, which suggests a low exposure to this mycotoxin, making it not possible to observe liver changes at plasma levels.

The use of blood creatinine is a fundamental indicator of renal damage, and the increase in the levels of urea and creatinine are indicative of nephrotoxicity (MIR; DWIVEDI, 2010; STOEV et al., 2012). Current research also evaluated blood markers of kidney damage (Table 1), and the mean values of urea (42.49 ± 16.76 mg/dL) and creatinine (0.84 ± 0.56 mg/dL) were within the normal range disclosed in the test kit (10-50 mg/dL; 0.5 a 1.5 mg/dL, respectively). Ochratoxicosis in pigs reveal an increase in serum levels of many blood components, including urea and creatinine, which is related to nephrotoxicity (MIR; DWIVEDI, 2010; STOEV et al., 2012). The researches using several animal species, as rats, pigs and also in eggs, demonstrate changes in the values of blood parameters between 1-2 months of exposure to OTA in a dose-time dependent way (ABDU; ALI; ANSARI, 2011; FROHLICH; MARQUARDT; OMINSKI, 1991; HASSAN et al., 2006; MIR; DWIVEDI, 2010; PLEADIN et al., 2012). Although data from animal experiments demonstrate a

correlation between blood levels of urea/creatinine and OTA, there was no correlation in this study with human samples: OTA x urea r = 0.118 and OTA x creatinine r = 0.09907.

When the samples were organized by plasma levels of OTA (pg/mL), most positive samples were below the limit of quantification or contaminated with OTA up to 700 pg/mL (Fig. 2A). Even after subdividing the results (Fig. 2B), it was not possible to observe a correlation with the parameters of kidney or liver damage and the plasma levels of OTA ($r < 0.5$).

As the experimental nephro and hepatotoxic effects are dose-time dependents of OTA, possibly the highest contamination observed in this study are due to temporary and not continuous exposures to OTA. In addition, most participants had low plasma levels of OTA and and the intake is below the necessary levels required for OTA to exert its nephro and hepatotoxic effects, possibly due to the low contamination of food products, as mentioned above.

Conclusion

Current research showed that OTA was detected in 55% of plasma samples analyzed, not differing between gender, age or micro northern region of Paraná state, Brazil. The participants' estimated intake is way below the limits established by the international regulating agencies and, possibly due to this, there was no correlation between OTA and changes in biochemical parameters of liver and kidney damage. This was the first study conducted in Brazil to analyze human plasma levels of OTA, requiring further researches in other regions, as Brazil is a very large country and has a diversity of food crops, as well as are necessary follow-up studies in several periods.

Conflict of interest statement:

The authors have no financial conflict of interest.

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Figure 1. Standard curve of the indirect competitive enzyme immunoassay for OTA, with mean calculated from 4 curves. Limit of detection 0.165 ng/mL; Limit of quantitation 0.371 ng/mL.

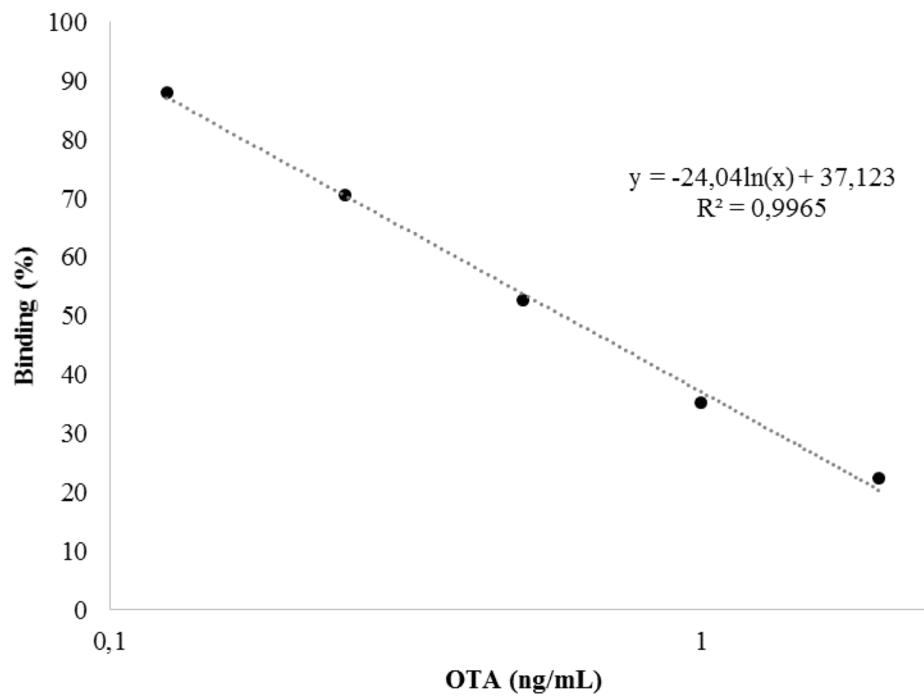


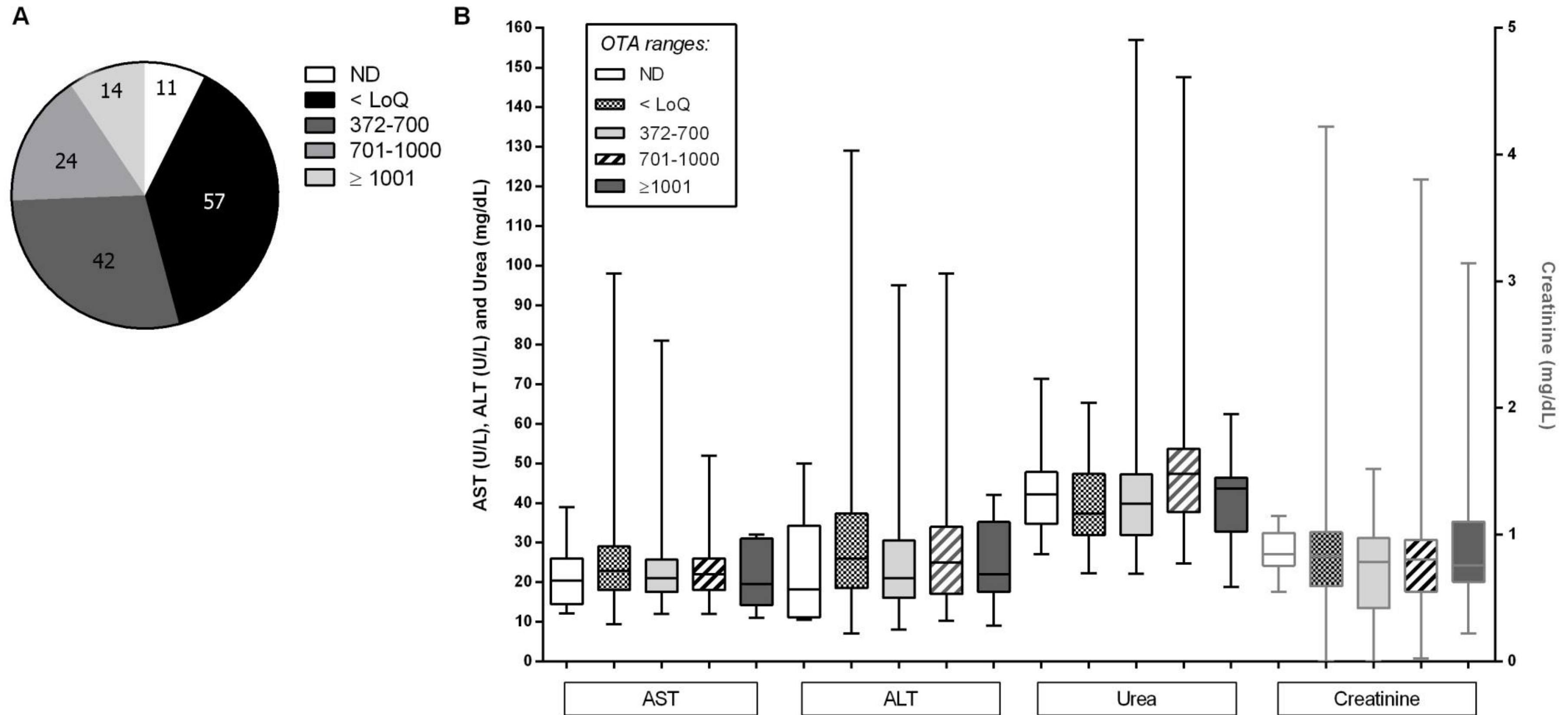
Table 1. Research results: number of samples, plasma levels of OTA, estimated intake and biochemical markers of liver and kidney damage.

ITEM ANALYZED	TOTAL	FEMALE	MALES
Number of samples analyzed	149	85	64
Number of samples positive for OTA	81	50	31
Ages (mean ± SD)	52.13 ± 15.42	49.8 ± 15.8	53.95 ±16
Limit of Detection (pg/mL)	165	----	----
Limit of Quantitation (pg/mL)	371	----	----
Mean ± SD, pg/mL(positives only)	733.6 ± 296.04	741 ± 304.21	722 ± 286,9
Range (pg/mL)	372.73 – 1584.6	372.7 – 1584.6	375 - 1489
Mean daily OTA intake (pg/kg bw)			
Equation 1, using CF 1.34[a]	983.1 ± 396.7	992.8 ± 407.6	967.4 ± 410.3
Equation 2, using CF 1.97[b]	1445.3 ± 583.2	1459.5 ± 599.3	1422.3 ± 565.3
AST (mean ± SD, U/L)	21.1 ± 5.54	20.65 ± 4.96	21.69 ± 6.23
ALT (mean ± SD, U/L)	21.81 ± 7.62	19.62 ± 6.26	24.43 ± 8.32
Urea (mean ± SD, mg/dL)	42.49 ± 16.76	43.01 ± 15.56	41.78 ± 18.38
Creatinine (mean ± SD, mg/dL)	0.84 ± 0.56	0.84 ± 0.60	0.83 ± 0.52

[a] Conversion factors (CF) taken from: Hagelberg, Hult and Fuchs, 1989; Breitholtz et al. 1991.
[b] Conversion factors (CF) taken from: Studer-Rohr, Schlatter, Dietrich, 2000; Miraglia Brera, Colatosti, 1996.

Reference values:
AST: male: 11 - 41 U/L female: 11 – 39 U/L
AST: male: 7 – 52 U/L female: 7 – 49 U/L
Urea: 10 – 52 mg/dL
Creatinine: male: 0.6 1 1.5 mg/dL female:0.5 – 1.3 mg/dL

Figure 2. (A) Percentages of plasma samples in sub-groups organized by plasma levels of OTA (pg/mL) determined by ic-ELISA: ND (7,4%), < LoQ (38,4%), 372-700 (28,5%), 701-1000 (16,2%), >1001 (9,4%). (B) Distribution of the biochemical parameters of liver and kidney damage according to the plasma levels of OTA (pg/mL).



ND: not detected (below the limit of detection)
< LoQ: positive results, which were below the limit of quantitation, but above the limit of detection.

5. ARTIGO B: Cytotoxic effect of Ochratoxin A, Fumonisin B_1 and their combination on Jurkat and P3U1 cell lines

Abstract

Contamination of food and foodstuffs by mycotoxins is one of the problems concerning human and animal health, which is also extremely harmful to the economy. The mycotoxins can have toxic potential, causing acute and chronic effects in humans and animals, such as immune toxicity and carcinogenicity. In this research, the cytotoxic effects of ochratoxin A (OTA) and fumonisin B_1 (FB_1) were assessed using Jurkat and P3U1 cells. These cells were exposed to different concentrations of OTA (0, 15, 30 and 90 µg/mL) for 30 min, 1 h, 2 h, 4 h, 8 h and 24 h, and of FB_1 (0, 30, 90 µg/mL) for 24 and 48 h, after which they were analyzed by the 3-(4,5-di-methylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide (MTT) assay and lactate dehydrogenase test (LDH). Cell viability was significantly reduced ($p < 0.05$) only when using 90 µg/mL of mycotoxins for 24 h, with OTA affecting both cell lines and FB_1 only Jurkat cells. The associated use of OTA and FB_1 did not enhance the cytotoxic effect. There was a strong correlation between MTT and LDH for Jurkat and P3U1 cells subjected to OTA ($r = 0.75$ and 0.931, respectively). Immunocytochemistry analysis by using monoclonal antibody (mAb) to OTA confirmed the decrease of viability of P3U1 and Jurkat cells treated with OTA, with destruction of the plasma membrane and release of cytosol components marked with peroxidase substrate. These *in vitro* results suggest the dose and time dependence of P3U1 and Jurkat cells cytotoxicity with OTA and association of OTA and FB_1 does not enhance the cytotoxic effect.

Keywords: Combined toxicity; Cytotoxicity; FB_1; Immunocytochemistry; OTA.

Introduction

Mycotoxins are toxic secondary metabolites of many fungi, with over 300 known types and their effects are at least partially characterized. These toxins are of great interest with regard to human and animal health, because according to estimates of the Food and Agriculture Organization (FAO) of the United Nations, approximately 25% of global food production is contaminated with at least one mycotoxin.

Ochratoxins are metabolites of several species of *Penicillium* and *Aspergillus*, like *A. carbonarius, A. ochraceus, A. westerdijkiae* and *Aspergillus* section *Nigri,* with many

naturally occurring isoforms, which are frequently co-produced. The largest and most toxic compound is ochratoxin A (OTA), which has been associated with many human and animal diseases, including Balkan nephropathy, urothelial tumors and nephropathy induced by mycotoxins in pigs (STOEV, 1998; STOEV; HALD; MANTLE, 1998; TATU et al., 1998; WAFA et al., 1998; PERAICA et al., 1999; PFOHL-LESZKOWICZ et al., 2002). OTA's mechanism of action has been a controversial topic (MANDERVILLE, 2005; TURESKY, 2005). OTA is known to promote oxidative damage to DNA on mammal cells (GAUTIER et al., 2001; KAMP et al., 2005a, 2005b), which causes cytotoxicity (KAMP et al., 2005b; ARBILLAGA et al., 2007; ALI et al., 2011) and relates to OTA's carcinogenicity (ARBILLAGA et al., 2007; MARIN-KUAN et al., 2011). This suggests that OTA is not a direct genotoxic carcinogen (MARIN-KUAN et al., 2011). However the International Agency for Research on Cancer (IARC) classified OTA as a class 2B , i.e. a compound possibly carcinogenic to humans.

Fumonisin (B_1 and B_2), metabolites from *Fusarium proliferatum* and *F. verticillioides*, are cancer promoters that have a long hydrocarbon chain (similar to sphinganine and sphingosine), which has a main role on its toxicity (WANG et al., 1992). Fumonisin B1 (FB_1) was shown to be a tumor promoter in rats (GELDERBLOM et al., 1988; ABEL; GELDERBLOM, 1998), to cause equine leukoencephalomalacia (MARASAS et al., 1988) and pulmonary edema in pigs (HARRISON et al., 1990). It is suggested that FB_1 exerts toxic effects due to its structural analogy with sphingoid bases and inhibits both DNA and protein synthesis (HUMPF et al., 1998). In addition, it promotes oxidative stress, induces DNA fragmentation and cell cycle arrest (ABADO-BECOGNEE et al., 1998; ABEL; GELDERBLOM, 1998; MOBIO et al., 2000, 2003).

As both these mycotoxins are frequent in many food and may cause significant damages to health, current research evaluated the cytotoxicity of OTA and FB_1, separately and in association, using Jurkat and P3U1 cell lines.

Material and Methods

Chemicals

FB_1 was purchased from Sigma (32936, Sigma Chemical Co., St. Louis, MO, USA) and OTA from Santa Cruz Biotechnology (sc-202749A, Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Cell culture

Mouse myeloma P3U1 cell line (P3U1) and human lymphoblastoid Jurkat T cell line (Jurkat) were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco Life Technologies, Rockville, MD, USA) and cultivated under standard conditions (37 °C, with 5% CO_2 atmosphere). Cell number and viability were assessed by trypan blue dye exclusion. For all experiments, cells were cultured at 2 x 10^6 /mL in flat-bottom 96-well plates (TPP® tissue culture plates, Sigma Chemical Co., St. Louis, MO, USA). Mycotoxins were dissolved in 0.15 M phosphate buffered saline (PBS)/methanol (9:1) and diluted in supplemented RPMI-1640 medium.

Production of MAb anti-OTA

The cell line anti-OTA used was Anti-OTA.7 MAb, cross-reactivity of 79.4 % for OTC, prepared at Science University of Tokyo, Japan (KAWAMURA et al., 1989). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% L-glutamine. The supernatant was collected and precipitated with saturated solution of ammonium sulfate [$(NH_4)_2SO_4$]. The precipitate was centrifuged (30 min, 2,500 rpm), redissolved in PBS and dialyzed against it. The IgG antibodies were purified by Protein A Antibody Affinity Chromatography (Affi-Prep® protein A, cat n° 156-0005, Bio-Rad Laboratories, Hercules, CA, USA) and the protein concentration determined at 280 nm.

MTT assay

P3U1 and Jurkat cells were seeded at plating density of 2 x 10^6 cells/mL (50 µL/well) and treated with OTA (0, 15, 30 and 90 µg/mL, for 30 min, 1 h, 2 h, 4 h, 8 h and 24 h), FB_1 (0, 30, 90 µg/mL for 24 h and 48 h) or OTA+FB_1 combinations (Control, 15+0, 15+15, 15+30, 15+90, 30+0, 30+15, 30+30, 30+90, 90+0, 90+15, 90+30, 90+90 µg/mL, for 24 and 48 h). Control cells were treated with methanol at 0.5% final concentration. At the end of the treatments, 10 µL of MTT reagent was added in each well for 4 hours, followed by 200 µL of dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and the precipitate was dissolved. The absorbance was measured at 550 nm in a Multiskan EX Reader (Labsystems, Helsinki, Finland). The results were expressed as percentage of control values.

Lactate Dehydrogenase (LDH) assay

After OTA treatments, degrees of cell death were assessed by measuring the activity of LDH released into the culture medium, according to the manufacturer's instructions (743161 LDH-Lact. Desidrogen. kit 2x30 mL, Laborclin Produtos para Laboratório Ltda., Pinhais, PR, BR). Aliquots of cell culture medium (15 µL) were incubated at room temperature in the presence of NADH + sodium pyruvate. Rates of NAD^+ formation were read at 340 nm using a microplate spectrophotometer (VICTOR™ *X3* Multilabel Plate Reader, Perkin Elmer Inc., Waltham, MA, USA).

Immunocytochemistry

Jurkat and P3U1 cells treated with OTA (90 µg/mL, 24 h) were fixed on slides sensitized with poly-L-lysine, fixed with acetone for 10 min, washed with PBS followed by blockade for 30 min at room temperature with normal horse serum (PK4002, VECTASTAIN® ABC Reagent - mouse IgG, Vector Laboratories Inc., Bulingame, CA, USA). Excess blocking was removed and slides were incubated with primary antibody anti-OTA for 1 h at room temperature, followed by biotinylated secondary antibody and reagentes A and B from the kit (1 h, room temperature). Cells were then incubated for 30 min with diaminobenzidine (DAB) peroxidase substrate solution (Liquid DAB+ K3467, Dako North America, Inc., Carpinteria, CA, USA). The reaction was stopped using running water and cells were counterstained with Harris hematoxylin for 20 seconds, followed by baths with alcohol 70, 80, 100% and xylene. The slides were examined under an optical microscope and pictures were taken at 40 and 100x magnifications.

Statistical Analysis

Data were analysed by GraphPad Prism 6.0 for Windows (GraphPad Software, Inc, La Jolla, CA, USA, www.graphpad.com) by T-test or one-way ANOVA with Tukey's post hoc test. Significance was defined as p-value ≤ 0.05. Spearman's correlation was defined as strong when $r \geq 0.75$.

Results

Dose and Time-response of OTA and FB_1

After completion of the exposure protocols, cell viability was evaluated by the MTT assay, demonstrating a significant reduction ($p < 0.001$) only after the use of 90 µg/mL for 24 h on both cell lines with OTA (Fig. 1, A-B) and on Jurkat cells with FB_1 (Fig. 1C).

Dosage of LDH of the culture supernatant evaluates the release of intracellular LDH and not cell proliferation, as does the MTT assay. Therefore, the dosage was employed to confirm cell death and corroborate the decrease in cell viability of Jurkat and P3U1 cells after the use of 90 µg/mL of OTA for 24 h (Fig. 2). By Spearman's correlation between MTT x LDH, there was a strong correlation for Jurkat cells ($r = 0.749$) and P3U1 cells ($r = 0.931$).

Cytotoxicity of OTA and FB_1 association on Jurkat and P3U1 cells

Figure 3 demonstrates the cell viability after exposure to different combinations of OTA and FB_1 for 24 hours, but there was no potentiation of the cytotoxic effects.

Immunocytochemistry

Taking into consideration the cytotoxic potential of OTA, immunocytochemistry was employed to evaluate this effect and the places where this mycotoxin may bind to the cell. As well as demonstrated by the MTT and LDH results, there was a marked decrease in viability of Jurkat and P3U1 cells treated with 90 µg/mL of OTA, when compared to control cells without treatment (Fig. 4). It was also possible to observe the increase in intracellular granules labeled with peroxidase substrate, indicating the presence of OTA in these cells, besides an extensive cell destruction with the release of cytosol to the extracellular medium.

Discussion

The cytotoxicity of OTA has been investigated with different cell lines by many authors: mammalian cells (KAMP et al., 2005b); Vero, C6 glioma and caco-2 cells (CREPPY et al., 2004); human hepatoma cell line (BALDI et al., 2004; CREPPY et al., 2004; RENZULLI et al., 2004; HUNDHAUSEN et al., 2005); human leukocytes (ODHAV;

ADAM; BHOOLA, 2008); porcine renal cell line (HEUSSNER; DIETRICH; O'BRIEN, 2006); embryonic cells (HONG et al., 2000); and neural stem/progenitors cells (SAVA et al., 2007). To our knowledge, there is no data published about the cytotoxic action of OTA and FB_1 on P3U1 cell line. Therefore, current study investigated the effects of different doses and times of exposure to OTA and FB_1 on this cell lineage.

P3U1 cells had their viability reduced only after 24 h exposure to 90 µg/mL of OTA (Fig. 1B). This is in agreement with the data from other cell lines, but there is a wide variation of responses of different lineages exposed to OTA. Kamp et al. (2005b) demonstrated that V79 and CV-1 cells had an IC of 2 µM/L for OTA within 24 hours of exposure. Other authors worked with human hepatocellular liver carcinoma cell line (HepG2), and observed the same pattern of cytotoxicity to OTA, with an IC_{50} of 35 µM/L after 48 hours and of 10 µM/L after 72 hours (BALDI et al., 2004; CREPPY et al., 2004; RENZULLI et al., 2004; HUNDHAUSEN et al., 2005). Using human leucocytes, Odhav, Adam and Bhoola (2008) observed an OTA IC_{50} of 50 µg/mL for an exposure time of 23 hours. Baldi et al. (2004) evaluated the effect of OTA on several cell lines after 24 and 48 hours of exposure and, like other authors, they also observed a response that was time and dose-dependent for all lineages, but with different half lethal concentrations (IC_{50}): mouse liver hepatocytes-12 (AML-12) was the most resistant ($IC_{50} > 40$µg/mL) and bovine mammary epithelium (BME-UV1) the most sensitive ($IC_{50} = 0,8$ µg/mL) after 24 hour exposure.

Jurkat cells were another lineage investigated in the current research, which turned out to be more resistant to the action of OTA than P3U1 cell line (Fig. 1A). Although in some aspects these results corroborate the data found in the literature, they show differences in the pattern of response to the treatment with OTA. There was a dose-time-dependent response, because when cell lines were exposed to different OTA concentrations (0, 30, 90 µg/mL) for 24 and 48 hours, there was an increase in cytotoxicity (Fig. 3, A-B).

When exposed to different concentrations of FB_1, there was no reduction in the viability of Jurkat cells in a dose-dependent manner, because there was just a small effect only with 90 µg/mL of FB_1 for 24 hours (Fig. 1C). After 48 hours of exposure, this effect was no longer seen, suggesting that the lymphoproliferative action of FB_1 predominates after some time of exposure, as already seen by other authors (TAJIMA et al., 2002; LUONGO et al., 2006; SEVERINO et al., 2008) (Fig. 3B). Nevertheless, when the P3U1 cells were exposed to FB_1, it did not demonstrate a cytotoxic effect and neither affected lymphoproliferation (Fig. 1D). Many researchers evaluated the effect of FB_1 on several cell lines and it seems there is no pattern of action: depending on the cell line, the mycotoxin concentration and duration of

exposure, FB_1 may either promote cell proliferation (TAJIMA et al., 2002; LUONGO et al., 2006; SEVERINO et al., 2008), as well as decrease cell viability (RUMORA et al., 2002; KOUADIO et al., 2007; KLARIĆ et al., 2008; ODHAV; ADAM; BHOOLA, 2008; FICHEUX; SIBIRIL; PARENT-MASSIN, 2012).

Another aspect analyzed in this research was the combined effect of the mycotoxins OTA and FB_1 on P3U1 and Jurkat cell lines. Differently from what is found in the literature, the use of OTA+FB_1 showed no synergistic effect on cell cytotoxicity nor an antagonist effect. The cytotoxicity caused by OTA on Jurkat and P3U1 cells was the same when associated with FB_1 (Fig. 3). The last decade has yielded much research on the combined effects of OTA and FB_1 (CREPPY et al., 2004; DOMIJAN et al., 2006; KLARIĆ et al., 2007, 2008, 2010; KLARIĆ; PEPELJNJAK; ROZGAJ, 2008; STOEV et al., 2012). In most cases, these toxins interacted in a synergistic or, at least, additive manner. Cytotoxic synergism was observed in rat brain glioma C6 cells, human intestinal Caco-2 cells and Vero cells exposed to low FB_1 and high OTA concentrations, because cytotoxicity was above additive effects concerning individual toxins (CREPPY et al., 2004). In pig studies, a mixture of OTA and FB_1 caused strong lesions in the kidneys, more pronounced changes in biochemical parameters and disturbances in humoral immune response in doses that corresponded to those found in cases of porcine nephropathy in Bulgaria and South Africa (STOEV et al., 2010a, 2010b, 2012).

A good overview and comparison of the combination effects in various systems is given by Speijers and Speijers (2004). Supported by the toxicity data of the individual toxins, it is likely that not only the individual toxins show large differences depending on species, sex, *in vivo* or *in vitro* exposure system, but also the differences in combination toxicity are probably influenced by the same factors.

Differences in the specific IC_{50} reported can probably be attributed to the use of different cell lines, different endpoints (e.g., MTT reduction, neutral red uptake, cell counting or LDH release) or to the presence or absence of serum in the culture medium (BONDY; ARMSTRONG, 1998; DIETRICH et al., 2001; HEUSSNER; DIETRICH; O'BRIEN, 2006). Therefore, we tested whether two of the most used methods to analyze cell viability (MTT and LDH) correlated, which resulted in strong correlation mainly with P3U1 line cells. Lobner (2000) compared LDH and MTT assay to quantify neuronal cell death and found that the level of damage was similar on both tests, while Fotakis and Timbrell (2006) compared four methods using human hepatoma and found different sensitivity for each assay, MTT being more sensitive to detect cytotoxicity than LDH. Another research investigated the

cytotoxicity on rat hepatocytes by LDH and trypan blue and found that the two methods were comparably sensitive (JAUREGUI et al., 1981). According to da Costa et al. (1999), MTT assay offers more advantages concerning speed, simplicity and precise quantitation over other tests of viability.

The use of immunocytochemistry after the exposure of the cells to OTA clearly demonstrates (Fig. 4): (1) rupture of the cells' membranes; (2) more intracellular granules recognized by the MAb anti-OTA; (3) decrease in the number of viable cells and; (4) increase of dead cells. Some plasma membranes from the control cells were lightly stained by the chromogenic substrate (DAB), which can lead to doubts about the specificity of the employed MAb. This concern is certainly extinguished because control slides prepared without MAb also had a small connection with DAB, suggesting that the substrate may bind alone weakly to cell membranes. Nevertheless, the staining of slides containing treated or control cells is visually distinct, ensuring the specificity of the MAb. This is the first research that demonstrates the recognition of MAb anti-OTA on cell lines exposed to this mycotoxin, and therefore more research is required.

In conclusion, we have shown that cytotoxic effects of OTA, FB_1 and their combinations can have different effects on cells, depending on the concentrations used and on the cell system studied. The cytotoxic effects of OTA on Jurkat and P3U1 cells were similar, whereas for FB_1, Jurkat cells were more sensitive. For the first time, we demonstrate immunocytochemistry images in which is possible to observe the recognition of MAb anti-OTA on cytoplasmic structures, and also the membrane rupture of the cells previously exposed to the action of OTA. Therefore, more research shall be performed in order to better understand these mechanisms.

Conflict of interest statement:

The authors have no financial conflict of interest.

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Figure 1. Cytotoxic effect measured by MTT assay. (A) Jurkat + OTA; (B) P3U1 + OTA; (C) Jurkat + FB_1; (D) P3U1 + FB_1. There was a significant decrease of cell viability (*) only when using 90 µg/mL of mycotoxins for 24 h, with OTA affecting both cell lines and FB_1 affecting only Jurkat cells ($p < 0.05$), even though P3U1 + FB_1 followed the same decreasing trend.

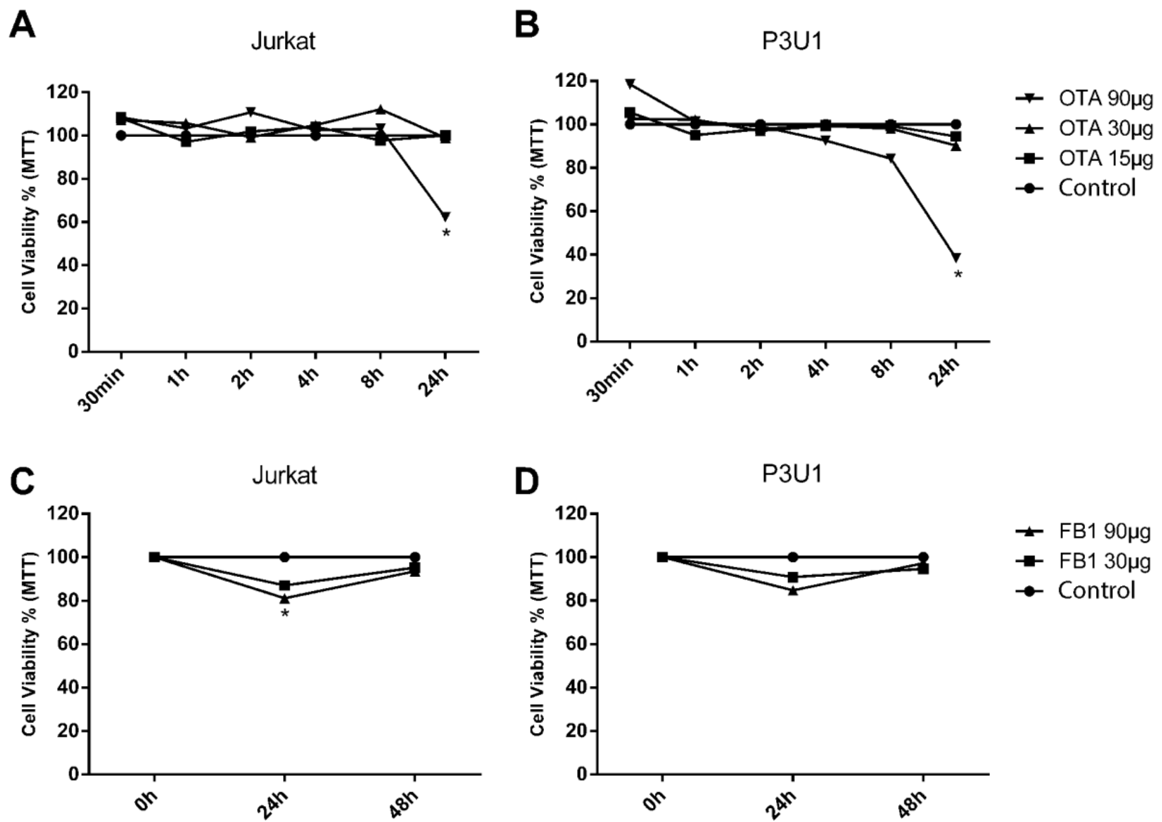


Figure 2. Comparison of the results from two different methods, MTT assay and LDH test, for the evaluation of the cytotoxicity after 24 hours of exposure. (A) Jurkat cells. (B) P3U1 cells. Both methods were equally efficient to detect OTA's cytotoxicity at 90 µg/mL.

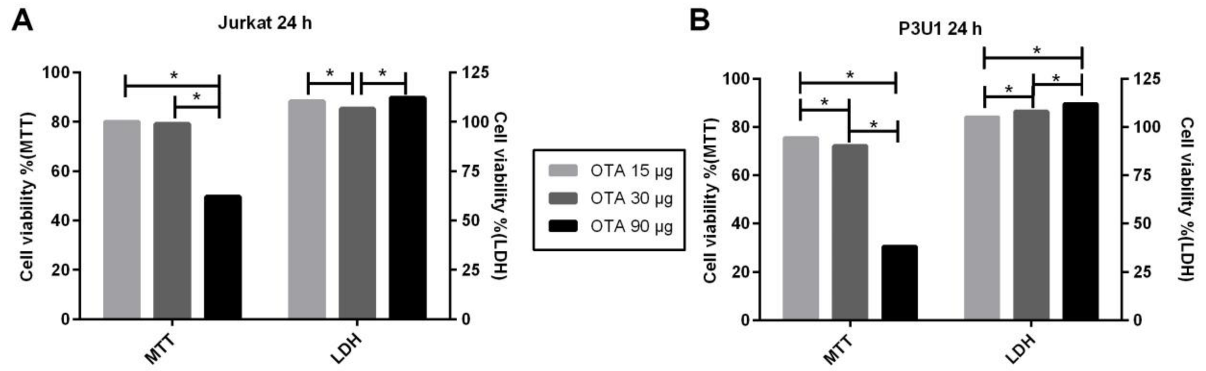


Figure 3. Cytotoxic effects of the exposure of cells to combinations of OTA and FB_1, measured by the MTT assay. (A, B) Effect after 24 and 48 hours on Jurkat (A, B) and P3U1 (C, D) cells. There was no synergistic effect with the use of OTA and FB_1 together.

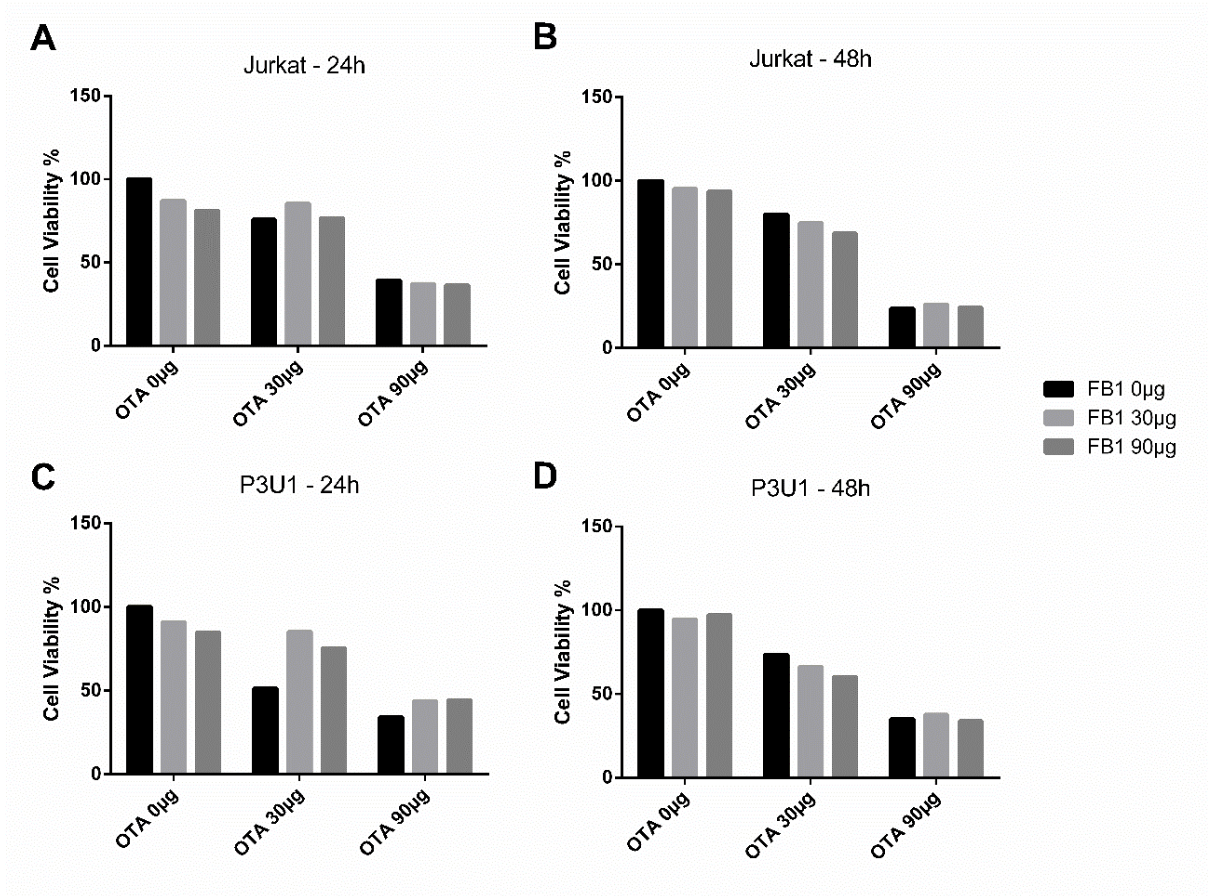
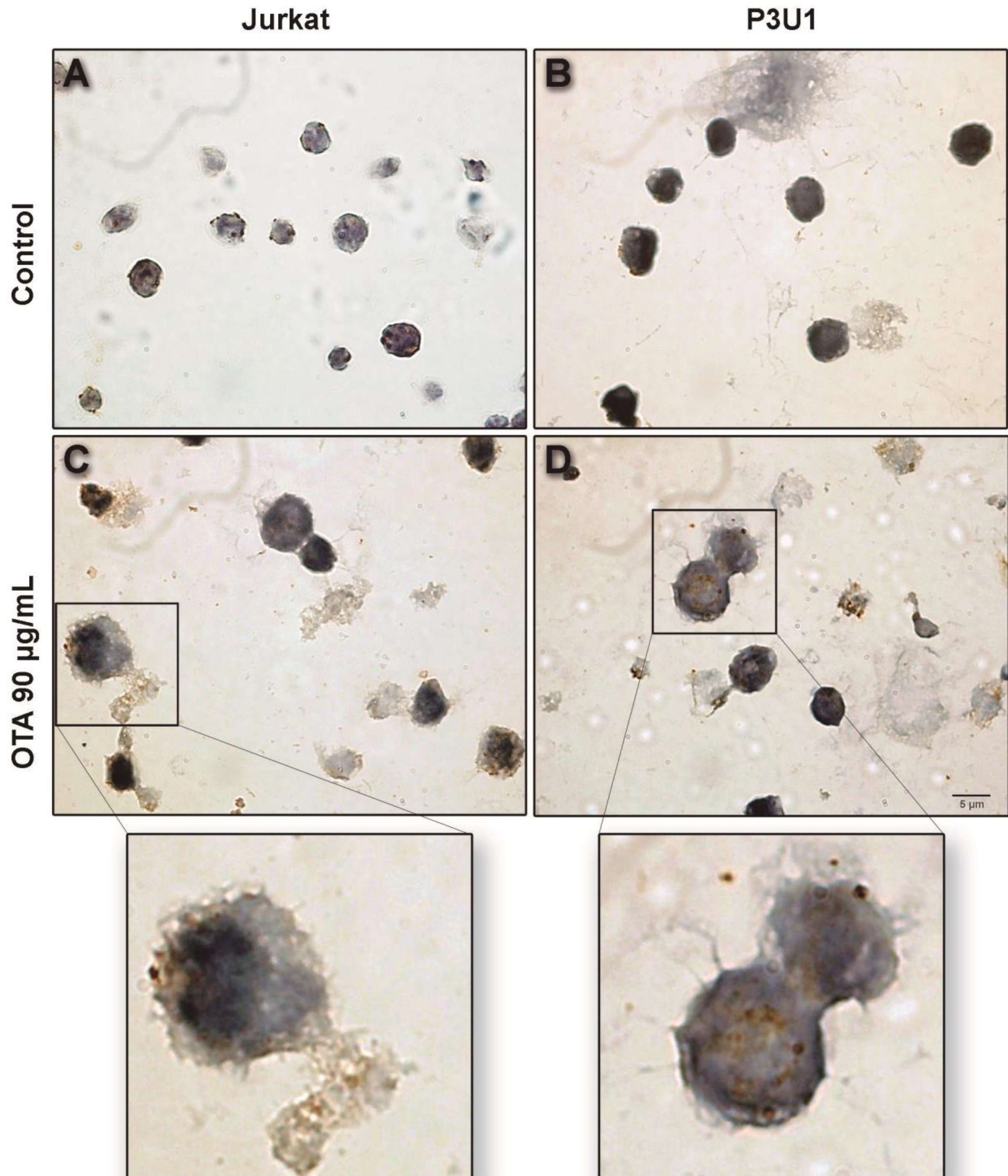


Figure 4. Immunocytochemistry performed with VECTASTAIN® ABC kit, photographed at 100x magnification. (A, B) Control cells. (C, D) Cells treated with 90 µg/mL of OTA. The treated group had a decrease in cell viability and showed the binding of Mab anti-OTA to several sites of the cells (zoomed areas). Scale bar: 5 µm.



6. CONCLUSÕES GERAIS

Com os dados obtidos conclui-se que o método de ELISA apresenta alta repetibilidade e reproducibilidade, permitindo a quantificação de OTA em amostras de plasmas humano. Este trabalho pioneiro no Brasil demonstra que 55% da comunidade analisada da região norte do Paraná encontra-se contaminada com baixa dose de OTA, sendo a estimativa de ingesta de OTA abaixo do limite estipulado pelos órgãos internacionais, o que pode explicar a falta de correlação com os parâmetros bioquímicos de lesão hepática e renal. As micotoxinas OTA, FB_1 e suas combinações apresentam efeitos citotóxicos dependentes de dose e de tempo sobre as linhagens celulares Jurkat e P3U1, *in vitro.*