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Pauline Christ Ledur

# AVALIAÇÃO DOS EFEITOS DE CURCUMINA E SILIMARINA SOBRE A TOXICIDADE INDUZIDA POR MICOTOXINAS *IN VITRO* E *IN VIVO*

Santa Maria, RS
2020

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor (a) em Farmacologia.**

Orientador: Prof. Dr. Janio Morais Santurio

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**Aprovado em 08 de Outubro de 2020:**

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**Janio Morais Santurio, Dr. (UFSM)**
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Santa Maria, RS
2020

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## RESUMO

# AVALIAÇÃO DOS EFEITOS DE CURCUMINA E SILIMARINA SOBRE A TOXICIDADE INDUZIDA POR MICOTOXINAS *IN VITRO* E *IN VIVO*

AUTOR: Pauline Christ Ledur
ORIENTADOR: Janio Morais Santurio

As micotoxinas são metabólitos secundários produzidos por fungos e representam uma preocupação mundial devido à sua distribuição global e aos efeitos prejudiciais causados pela sua ingestão. Esse problema se apresenta em sua forma mais dramática nos animais de produção, especialmente os suínos, que são considerados os animais de produção mais sensíveis aos efeitos das micotoxinas. Dessa forma, as micotoxinas têm se mostrado um grave problema econômico, sendo fundamental elucidar seus mecanismos toxicológicos e buscar alternativas que minimizem os danos por elas causados. Nesse sentido, têm-se demonstrado que compostos fenólicos e outros antioxidantes têm a capacidade de proteger e até mesmo neutralizar os danos causados por várias substâncias tóxicas, incluindo as micotoxinas. A curcumina e a silimarina são dois polifenóis conhecidos por sua forte atividade antioxidante extraídos das plantas *Curcuma longa* e *Silybum marianum*, respectivamente. Neste contexto, este estudo teve por objetivos: a) avaliar a toxicidade da ocratoxina A (OTA) em células mononucleares do sangue periférico de suínos e células de rim suíno da linhagem PK-15 através da avaliação da viabilidade celular, estresse oxidativo e atividade mitocondrial; b) avaliar o efeito protetor do pré-tratamento de curcumina e silimarina sobre células renais da linhagem PK-15 de suínos *in vitro* sobre a toxicidade induzida por OTA, fumonisina $B_1$ ($FB_1$) e deoxinivalenol (DON), por meio da avaliação de viabilidade celular, estresse oxidativo e apoptose; e c) investigar o efeito do pré-tratamento com curcumina e silimarina na prevenção da toxicidade aguda causada por $FB_1$ e OTA sobre o estresse oxidativo hepático e renal, bem como sobre biomarcadores sorológicos de função hepática e renal em camundongos *in vivo*. Foi observado que os efeitos tóxicos da OTA em células PK-15 e PBMCs suínas são bastante semelhantes, onde a exposição à OTA causa perda de viabilidade celular, aumenta o estresse oxidativo e causa inibição das enzimas superóxido dismutase e catalase, além de causar disfunção mitocondrial, inibindo o complexo I da cadeia transportadora de elétrons. O pré-tratamento com curcumina e silimarina se mostrou eficiente em proteger as células PK-15 de danos causados pelas micotoxinas; curcumina diminuiu a perda de viabilidade celular induzida por OTA, $FB_1$ e DON; inibiu a formação de espécies reativas de oxigênio desencadeadas por $FB_1$ e DON e diminuiu a apoptose em células expostas a DON. Já o pré-tratamento das células com silimarina diminuiu a perda de viabilidade celular induzida por OTA, $FB_1$ e DON; inibiu a formação de espécies reativas de oxigênio desencadeadas por $FB_1$ e DON e diminuiu a apoptose em células expostas a FB1 e DON. Na avaliação do efeito protetor de curcumina e silimarina sobre a toxicidade hepática e renal causada por OTA e $FB_1$ em camundongos, foi observado que ambos os compostos, tanto quando administrados isolados, como em combinação, foram capazes de mitigar os efeitos deletérios causados pelas micotoxinas, promovendo melhora na função hepática e renal e prevenindo o estresse oxidativo, além de evitarem dano tubular renal e necrose de hepatócitos causada pela exposição às micotoxinas OTA e $FB_1$. De maneira geral, os resultados deste estudo demonstraram que tanto a curcumina como a silimarina proporcionaram proteção contra os efeitos deletérios causados pela exposição à micotoxinas.



**Palavras-chave:** Toxicidade; Ocratoxina A; Fumonisina $B_1$; Deoxinivalenol; Curcumina; Silimarina.

## ABSTRACT

# EVALUATION OF THE EFFECTS OF CURCUMIN AND SILYMARIN ON THE TOXICITY INDUCED BY MYCOTOXINS *IN VITRO* AND *IN VIVO*

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Mycotoxins are secondary metabolites produced by fungi and represent a worldwide concern due to their global distribution and the harmful effects caused by their ingestion. This problem presents itself in its most dramatic form in farm animals, especially pigs, which are considered the farm animals most sensitive to the effects of mycotoxins. Thus, mycotoxins have been shown to be a serious economic problem, it is essential to elucidate their toxicological mechanisms and seek alternatives that minimize the damage caused by them. In this sense, phenolic compounds and other antioxidants have been shown to have the ability to protect and even neutralize the damage caused by various toxic substances, including mycotoxins. Curcumin and silymarin are two polyphenols known for their strong antioxidant activity extracted from the plants *Curcuma longa* and *Silybum marianum*, respectively. In this context, this study aimed to: a) evaluate the toxicity of ochratoxin A (OTA) in pig peripheral blood mononuclear cells and porcine kidney cells of the PK-15 cell line through the evaluation of cell viability, oxidative stress and mitochondrial activity ; b) to evaluate the protective effect of curcumin and silymarin pretreatment on PK-15 cells *in vitro* on the toxicity induced by OTA, fumonisin $B_1$ ($FB_1$) and deoxynivalenol (DON), via cellular viability assessment, oxidative stress and apoptosis; and c) to investigate the effect of pretreatment with curcumin and silymarin in the prevention of acute toxicity caused by $FB_1$ and OTA on hepatic and kidney oxidative stress, as well as on serological biomarkers of hepatic and kidney function in mice *in vivo*. We observed that the toxic effects of OTA in PK-15 cells and porcine PBMCs are quite similar, exposure to OTA causes loss of cell viability, increases oxidative stress and causes inhibition of the enzymes superoxide dismutase and catalase, in addition to causing mitochondrial dysfunction , inhibiting complex I of the electron transport chain. Pretreatment with curcumin and silymarin proved to be efficient in protecting PK-15 cells from damage caused by mycotoxins; curcumin decreased the loss of cell viability induced by OTA, $FB_1$ and DON; inhibited the formation of reactive oxygen species triggered by $FB_1$ and DON and decreased apoptosis in cells exposed to DON. The pretreatment of cells with silymarin decreased the loss of cell viability induced by OTA, $FB_1$ and DON; inhibited the formation of reactive oxygen species produced by $FB_1$ and DON and decreased apoptosis in cells exposed to $FB_1$ and DON. In the evaluation of the protective effects of curcumin and silymarin on liver and kidney toxicity caused by OTA and $FB_1$ in mice, it was observed that both compounds, administered alone or in combination, were able to mitigate the harmful effects caused by mycotoxins, promoting improvement in liver and kidney function and preventing oxidative stress, in addition to preventing kidney tubular damage and hepatocyte necrosis caused by exposure to the mycotoxins OTA and $FB_1$. In general, the results of this study demonstrated that both curcumin and silymarin provided protection against the harmful effects caused by exposure to mycotoxins.


**Key-words:** Toxicity; Ochratoxin A; Fumonisin $B_1$; Deoxynivalenol; Curcumin; Silymarin.

# LISTA DE ILUSTRAÇÕES

# SUMÁRIO

## APRESENTAÇÃO

Esta tese está estruturada em capítulos, sendo eles INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA, OBJETIVOS, PROTOCOLO EXPERIMENTAL I, II e III, DISCUSSÃO e CONCLUSÃO.

A INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA trata de todo o contexto desta tese e traz o referencial teórico que embasa este estudo. Nos OBJETIVOS estão citados o objetivo geral desta tese e os objetivos específicos referentes a cada um dos manuscritos produzidos.

A metodologia, resultados e discussão desta tese estão apresentados sob a forma de manuscritos, os quais encontram-se nos itens PROTOCOLO EXPERIMENTAL I, II e III, e estão estruturados de acordo com as normas das revistas científicas nos quais foram submetidos/ publicados.

Os capítulos DISCUSSÃO e CONCLUSÕES, encontrados no final desta tese, apresentam interpretações e comentários gerais a respeito dos resultados demonstrados nos manuscritos contidos neste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem nos capítulos INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA e DISCUSSÃO, desta tese. As referências utilizadas para a composição dos manuscritos estão apresentadas ao final dos próprios manuscritos.

# 1 INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

## 1.1 MICOTOXINAS

Os fungos filamentosos produzem diversos metabólitos secundários, dentre os quais estão as micotoxinas, que muitas vezes contaminam produtos alimentícios como milho, trigo, amendoim, cevada, centeio, entre outros. As micotoxinas são consideradas compostos biológicos naturais de baixo peso molecular, alta lipossolubilidade, estáveis sob calor intenso e normalmente tóxicas (IHESHIULOR, 2011), que não tem importância bioquímica ou no desenvolvimento dos fungos que as produzem. Fungos toxicogênicos podem produzir um ou mais desses metabólitos secundários, mas sabe-se que nem todos os fungos filamentosos são toxicogênicos e nem todos os metabólitos secundários de fungos são tóxicos (HUSSEIN; BRASEL, 2001). É importante salientar que a maioria das micotoxinas apresenta grande estabilidade química, o que favorece a sua constância nos grãos e alimentos em geral (HUSSEIN; BRASEL, 2001).

Os fungos são os mais importantes patógenos de plantas e insetos, tendo importância menor nas infecções em vertebrados. As infecções por fungos em hospedeiros animais são genericamente denominadas micoses, enquanto que as doenças causadas pela exposição aos seus metabólitos tóxicos, seja por meio da dieta, inalação, contato com a pele, entre outras formas de exposição, são chamadas de micotoxicoses (BENNETT; KLICH, 2003). As micotoxicoses podem acometer tanto os animais quanto os humanos. A exposição humana às micotoxinas pode ocorrer através do consumo de alimentos contaminados, principalmente grãos, por inalação e contato direto com a pele, e está associada à ocorrência de casos de câncer e efeitos deletérios em recém-nascidos (SHUAIB et al., 2010; MARIN et al., 2013).

As micotoxicoses são caracterizadas por síndromes difusas, seus sintomas dependem do tipo de micotoxina, da quantidade de toxina e duração da exposição, da idade, estado de saúde e sexo do indivíduo, além de outros fatores ainda não muito compreendidos, como envolvimento genético, estado nutricional e interações com outras substâncias tóxicas. Dessa forma, a severidade da intoxicação por micotoxinas pode ser agravada por fatores como deficiência de vitaminas, desnutrição, abuso de drogas como o álcool, e doenças infecciosas, como por exemplo a hepatite. Por sua vez, as micotoxicoses podem aumentar a vulnerabilidade à doenças infecciosas, piorar os efeitos da desnutrição e interagir sinergicamente com outras toxinas. As micotoxinas podem causar lesões em vários órgãos, como fígado, rins, pulmão, tecido epitelial e sistema nervoso, dependendo do tipo de toxina.

O desenvolvimento de uma micotoxicose não depende do contato direto com o fungo produtor da toxina, sendo, portanto, um risco abiótico, mas com origem biótica (BENNETT; KLICH, 2003; MARIN et al., 2013).

Vários fatores biológicos, químicos e físicos contribuem para a ocorrência dos metabólitos secundários de fungos nos alimentos, dentre eles a temperatura, umidade, incidência pluviométrica, suscetibilidade da planta e composição dos grãos, danos por insetos ou danos mecânicos durante a colheita e armazenamento. Esses fatores podem afetar as culturas tanto no período pré-colheita, como durante e após a colheita, no transporte e armazenamento (HUSSEIN; BRASEL, 2001; MARROQUIN-CARDONA et al., 2014).

Nesse sentido, as condições climáticas e ambientais como temperatura e umidade podem favorecer o crescimento dos fungos ou a produção das micotoxinas, como exemplo, sabe-se que espécies de *Fusarium* costumam crescer em locais com temperaturas que variam de 26 – 28 ºC e com atividade de água maior que 0.88, enquanto *Aspergillus* cresce melhor em temperaturas mais elevadas, de até 30 ºC, sendo que a produção de aflatoxinas é aumentada com atividade de água mais elevada, entre 0.9 – 0.92 (KLICH, 2007; MOUSA et al., 2013; MARROQUIN-CARDONA et al., 2014). Por outro lado, ambientes mais secos, com atividade de água menor ou igual a 0.85 permitem o crescimento de *Aspergillus flavus* e *Aspergillus parasiticus*, além do fato que a seca, por si só, é um fator estressante para as plantas, podendo favorecer a contaminação por estes fungos, ocasionando em uma maior presença de micotoxinas (KEBEDE et al., 2012). As precipitações pluviométricas são outro fator importante na presença de micotoxinas nos alimentos. Chuvas severas no período de floração das plantas estão associadas com uma maior dispersão de *Fusarium* nas plantações de milho, por facilitar a entrada dos conídios nas espigas. Além disso, chuvas fora de época podem favorecer a produção de micotoxinas em grãos e cereais por forçarem a colheita e armazenamento precoce dessas culturas, favorecendo o desenvolvimento dos fungos e a consequente produção de micotoxinas (MARROQUIN-CARDONA et al., 2014).

Outro fator importante que pode influenciar o crescimento fúngico e produção de micotoxinas nos alimentos são as condições de armazenamento. Em condições ideais, o armazenamento deve ocorrer no momento apropriado após a maturação das plantas, porém sob certas circunstâncias, pode ocorrer prematuramente, como mencionado anteriormente, aumentando a probabilidade de contaminação por fungos. Por outro lado, para algumas amêndoas, a colheita e armazenamento antes da completa maturação na planta podem reduzir o risco de contaminação, por reduzir o tempo de exposição das amêndoas aos ataques de insetos e quebras, que abrem portas para a contaminação fúngica. Além disso, deve-se evitar

ao máximo danos mecânicos às sementes e grãos, bem como contato com o solo, visto que esporos fúngicos podem persistir no ambiente por meses. Também deve-se ter um controle rigoroso da umidade e implementar técnicas que possibilitem a retirada de grãos e amêndoas danificadas, para evitar a contaminação de toda a produção, visto que são os mais propensos à contaminação por fungos (MARROQUIN-CARDONA et al., 2014).

Os gêneros dos fungos *Aspergillus*, *Penicillium* e *Fusarium* são as mais frequentemente encontradas como invasoras de culturas de grãos. As principais micotoxinas identificadas em alimentos, de relevância agroeconômica e para a saúde pública são as aflatoxinas, ocratoxinas, tricotecenos, zearalenona e fumonisinas (MARIN et al., 2013).

As aflatoxinas são as micotoxinas mais frequentemente encontradas como contaminantes em alimentos, e são produzidas principalmente por *A. flavus* e *A. parasiticus* (BENNETT; KLICH, 2003). Dentre as aflatoxinas, a mais predominante é a aflatoxina $B_1$ ($AFB_1$), classificada no Grupo 1 de substâncias carcinogênicas em humanos. O principal alvo toxicológico da $AFB_1$ é o fígado. Os sintomas inicias de toxicidade aguda causada por $AFB_1$ incluem febre, mal-estar e anorexia, seguidos de dor abdominal, náusea e necrose hemorrágica do fígado e letargia, no entanto, casos de intoxicação aguda são raros. Já a toxicidade crônica resulta em imunossupressão, retardo no crescimento e carcinogênese (KUMAR et al., 2016). Os efeitos tóxicos das aflatoxinas dependem de fatores como idade, sexo, espécie e estado nutricional do organismo intoxicado (WILLIAMS et al., 2004).

Os tricotecenos são micotoxinas produzidas principalmente por fungos do gênero *Fusarium*. Essas toxinas são constituídas por anéis tricotecanos que apresentam uma ligação dupla entre os carbonos 9 e 10 e um grupamento epóxido nas posições 12 e 13 da estrutura. Os tricotecenos mais estudados são deoxinivalenol (DON), diacetoxiscirpenol (DAS) e toxina T-2, enquanto que os mais tóxicos são DAS e toxina T-2 (BENNETT; KLICH, 2003). Os tricotecenos causam inibição da síntese proteica, afetando a subunidade 60S do ribossomo, além de causarem interrupção no transporte de membrana e estresse oxidativo (HUSSEIN; BRASEL, 2001). Além disso, são conhecidos por causarem neurotoxicidade, imunossupressão e toxicidade renal (RICHARD, 2007).

A zearalenona é uma micotoxina estrogênica produzida por diferentes espécies do gênero *Fusarium*, sendo que *F. graminearum* é a espécie mais responsável pelos efeitos estrogênicos normalmente observados em animais de produção (HUSSEIN; BRASEL, 2001). Apesar de não ser classificada como carcinogênica em humanos, a zearalenona requer atenção pelos seus efeitos estrogênicos, uma vez que foram relatados casos de puberdade precoce em meninas associados à esta micotoxina devido à interação com receptores para o 17β-estradiol

(MASSART et al., 2008), pela produção de espécies reativas de oxigênio (EROs) (EL GOLLI BENNOUR et al., 2009) e efeitos anabólicos (MARROQUIN-CARDONA et al., 2014).

A ocratoxina A, a fumonisina $B_1$ e o deoxinivalenol, que também têm grande destaque pelos seus efeitos tóxicos na saúde humana e animal e pelas perdas econômicas na agricultura e pecuária, são o foco deste trabalho e serão descritas com maior detalhe a seguir.

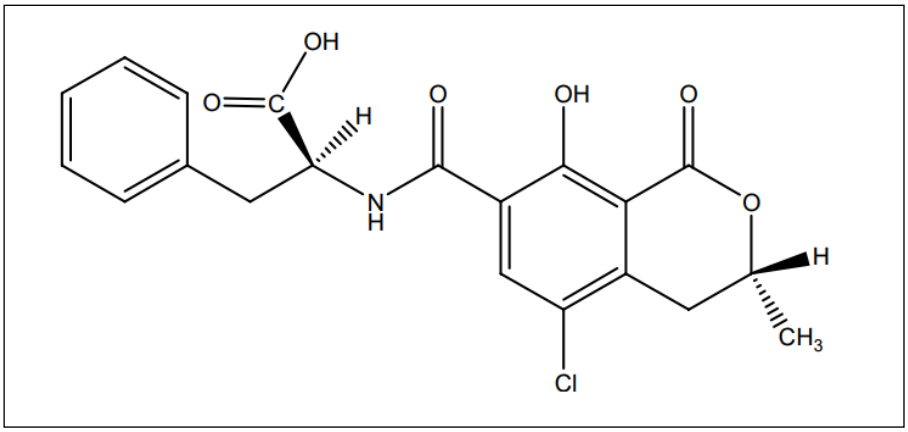
### 1.1.1 Ocratoxina A

A ocratoxina A (OTA) foi descrita pela primeira vez em 1965, tendo sido encontrada primeiramente em *Aspergillus ochraceus* (VAN DER MERWE et al., 1965). As ocratoxinas são produzidas por algumas espécies de *Aspergillus*, tais como *A. ochraceus* e *A. niger* e algumas espécies de *Penicillium*, principalmente *P. verrucosum*. A OTA é a mais prevalente e abundante entre as ocratoxinas, tendo sido descrita também como a de maior importância toxicológica (HUSSEIN; BRASEL, 2001).

A OTA é um importante contaminante na alimentação animal e em alimentos em geral, sendo frequentemente detectada em todos os tipos de cereais e derivados, além de café, cacau, uvas, vinho, soja, temperos, amêndoas e cerveja. Embora uma boa seleção dos produtos alimentícios e cuidados no transporte e armazenamento desses produtos possam diminuir muito a contaminação por OTA, ainda não parece possível evitar totalmente as contaminações por essa micotoxina (HEUSSNER; BINGLE, 2015). Por ter uma estrutura química estável (Fig. 1), a OTA não é muito afetada pelas temperaturas normalmente utilizadas no processamento de alimentos, mantendo-se constante mesmo quando submetida à altas temperaturas (BULLERMAN; BIANCHINI, 2007).

A OTA possui uma alta afinidade por proteínas, especialmente albumina, promovendo assim a bio-acumulação dessa micotoxina nos órgãos dos animais que consomem alimentos contaminados. Consequentemente, produtos desses animais, como carne, miudezas, leite e ovos, podem ser contaminados pela OTA (DUARTE et al., 2012). Estruturalmente, essa micotoxina é similar ao aminoácido fenilalanina (Phe), o que causa a inibição de várias enzimas que usam este aminoácido como substrato, especialmente a Phe-tRNA sintetase, o que pode resultar na diminuição da síntese proteica. Além disso, a OTA apresenta importante toxicidade mitocondrial, causando danos mitocondriais, estresse oxidativo, lipoperoxidação, além de interferir na fosforilação oxidativa (KUIPER-GOODMAN; SCOTT, 1989; KUIPER-GOODMAN et al., 2010).

Figura 1 – Estrutura química da ocratoxina A:




Fonte: EL KHOURY;ATOUI (2010).

A exposição à altas concentrações de OTA tem sido associada à disfunção e carcinogênese renal em humanos. Os rins são os principais órgãos afetados pela OTA, que é reconhecida como uma potente nefrotoxina. Estudos têm demonstrado que a exposição à OTA resulta na destruição das células do epitélio renal tubular, causando progressiva falência renal. Essa doença é particularmente proeminente na região dos Bálcãs e é conhecida como nefropatia endêmica dos Bálcãs (RAGHUBEER et al., 2015). A OTA também tem sido relacionada ao carcinoma urotelial do trato urinário superior e foi classificada como carcinógeno do Grupo 2B pela IARC (*International Agency for Research on Cancer*) (ZHU et al., 2017).

Além dos sintomas renais, a OTA pode afetar outros sistemas do organismo. Essa micotoxina pode atravessar a placenta e foi descrita toxicidade em embriões em ratos e camundongos. Estudos em animais têm mostrado que OTA também é imunotóxica, sendo a atividade imunossupressora caracterizada por redução no tamanho do timo, baço e linfonodos, depressão das respostas de anticorpos, alterações no número e função das células imunes e modulação da produção de citocinas. Além disso, mostrou-se que a OTA está associada a efeitos neurológicos adversos no cerebelo e hipocampo. Além disso, OTA pode ser encontrada no leite, o que pode representar uma fonte de exposição para filhotes (HOPE; HOPE, 2012)

O perfil toxicológico da OTA tem sido extensivamente investigado, mostrando efeito nefrotóxico, hepatotóxico, teratogênico, imunossupressor e genotóxico em várias espécies

animais e em estudos *in vitro*, sendo a toxicidade e carcinogênese renal os principais efeitos encontrados, sem ligação direta com genotoxicidade, mas envolvendo diferentes fatores epigenéticos ligados ao estresse oxidativo, proliferação celular e interferência na sinalização e divisão celular (ZHU et al., 2017). Os mecanismos de toxicidade e carcinogênese de OTA foram associados com indução de estresse oxidativo, inflamação e apoptose celular (RAGHUBEER et al., 2017), autofagia e inibição da síntese proteica (DA SILVA et al., 2018).

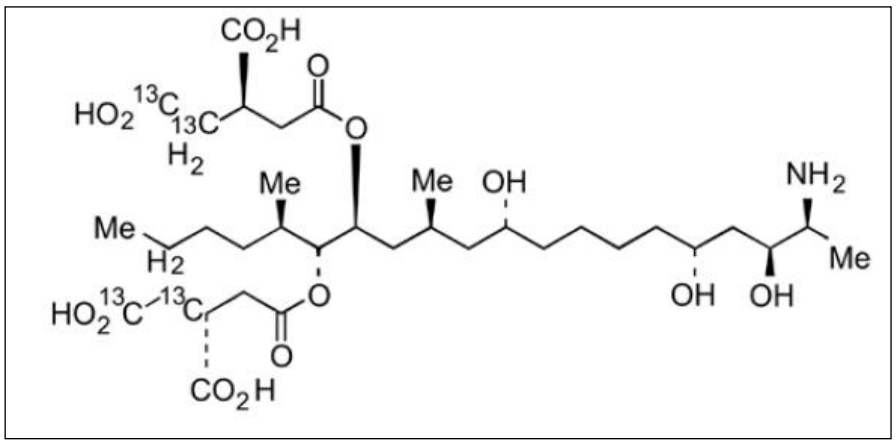
A formação de EROs vem sendo indicada como mecanismo causal da toxicidade da OTA, tendo sido propostos por estudos *in vitro* e *in vivo*. De maneira geral, foi demonstrado que a OTA pode causar danos oxidativos via geração de radicais hidroxila por meio da reação de Fenton, por meio da ativação da NADPH-citocromo P450, inibição da ativação do gene Nrf2 e transcrição gênica, além de causar diminuição na expressão das enzimas antioxidantes GPx, CAT, SOD e GR e aumento dos níveis de MDA (BOESCH-SAADATMANDI et al., 2008; BHAT et al., 2016; DA SILVA et al., 2018).

### 1.1.2 Fumonisina B1

Intoxicações por fumonisinas normalmente ocorrem através da ingestão de alimentos contaminados por *Fusarium verticillioides*. A fumonisina $B_1$ ($FB_1$) é a mais abundante e tem maior importância toxicológica. Em humanos a $FB_1$ tem sido relacionada ao câncer de esôfago, tumores hepáticos, estímulo e supressão do sistema imune, defeitos nos tubos neurais, nefrotoxicidade e outras anormalidades (CHU; LI, 1994; VOSS et al., 2002; MARASAS et al., 2004; GRENIER et al., 2011). Outros estudos também apontam a associação entre $FB_1$ e nanismo infantil (KIMANYA et al., 2010). A $FB_1$ apresenta efeitos hepato e nefrocarcinogênicos em ratos e é classificada como Grupo 2B em carcinogênese (possivelmente carcinogênica a humanos) pela Organização Mundial da Saúde (OMS) (PERSSON et al., 2012).

A fumonisina apresenta uma estrutura molecular similar a da esfinganina e da esfingosina, ambas componentes celulares do esqueleto de carbono dos esfingolipídios, afetando, por isso, os níveis celulares destes componentes. A estrutura química da $FB_1$ pode ser observada na figura 2.

Figura 2 – Estrutura química da fumonisina $B_1$:




Fonte: AHMED ADAM et al. (2017).

Um dos primeiros eventos que ocorrem nos órgãos atingidos pela $FB_1$ em organismos expostos a essa micotoxina é a apoptose, que pode ser consequência da inibição da síntese de ceramida e alterações no metabolismo de esfingolipídios ou a inibição da proteína quinase C, chave na sinalização da apoptose (SMITH et al., 1997; SEEFELDER et al., 2003). A regulação da biossíntese de esfingolipídios é essencial para a célula, visto que seus produtos afetam o metabolismo celular. A ceramida e a esfingosina são citotóxicas, inibem o crescimento e podem induzir apoptose precoce. No entanto, a esfingosina pode ser fosforilada pela esfingosina quinase formando um mitógeno potente que antagoniza o efeito apoptótico mediado pela ceramida. Dessa forma sugere-se que o balanço entre níveis intracelulares de ceramida e esfingosina-1-fosfato podem determinar a sobrevivência da célula (CUVILLIER, 2002).

Estudos *in vitro* e *in vivo* também têm demonstrado o potencial da $FB_1$ de promover estresse oxidativo e consequente aumento nos níveis celulares de EROs e seus efeitos citotóxicos, e indução de apoptose (DOMIJAN et al., 2015; ABBES et al., 2016). Porém, no caso dessa micotoxina, alguns estudos sugerem que o estresse oxidativo parece surgir como consequência e não como mecanismo causal da toxicidade (WANG et al., 2016), enquanto outros afirmam que a $FB_1$ é capaz de promover um aumento de radicais livres e acelerar as reações que levam à peroxidação lipídica das membranas (HASSAN et al., 2015). Porém, estudos realizados em diferentes modelos animais demonstraram que a $FB_1$ é capaz de modular a expressão de enzimas do sistema antioxidante como a SOD e GPx, bem como

diminuir os níveis de GSH e aumentar os níveis de MDA (DOMIJAN, PERAICA, et al., 2007; ABBES et al., 2016; DA SILVA et al., 2018).

O aumento na produção de EROs induzido por $FB_1$ também tem sido associado com inibição de síntese e fragmentação do DNA (WANG et al., 2016), inibição de síntese proteica (DOMIJAN, PERAICA, et al., 2007), dano mitocondrial com consequente ruptura da homeostase de cálcio e ativação da caspase-3, indução da atividade do citocromo P450 com aumento no metabolismo do ácido araquidônico e modulação da resposta inflamatória, além de ativar vias de sinalização da apoptose (ABBES et al., 2016; MARY et al., 2017; DA SILVA et al., 2018).

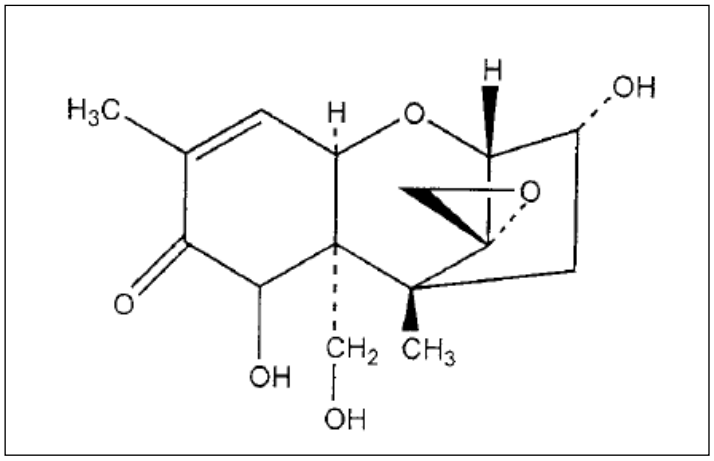
### 1.1.3 Deoxinivalenol

O deoxinivalenol (DON) é uma micotoxina do grupo dos tricotecenos. Os tricotecenos constituem uma família de metabólitos composta por mais de sessenta substâncias produzidas por fungos de vários gêneros, incluindo *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium*, entre outros (BENNETT; KLICH, 2003). Os tricotecenos mais amplamente estudados são diacetoxyscirpenol, toxina T-2 e DON, produzidas por espécies do gênero *Fusarium*. DON é um tricoteceno do tipo B e é menos tóxica que a toxina T-2 e vários outros tricotecenos, porém é a mais amplamente distribuída, comumente encontrada em cevada, milho, trigo, aveia e em rações mistas, sendo produzida majoritariamente na fase de pré-colheita, porém também pode ocorrer contaminação secundária no armazenamento dos cereais (BENNETT; KLICH, 2003; MA; GUO, 2008; WU et al., 2017).

Estruturalmente, DON é um composto polar e contém três grupos hidroxila (-OH) livres (Figura 3), que estão diretamente relacionados à sua toxicidade (MISHRA et al., 2014). Os efeitos fisiológicos da intoxicação por DON incluem vômito e anorexia, alteração na função intestinal e sistema imune, marcado pela diminuição na absorção de nutrientes e um aumento na suscetibilidade a infecções e doenças crônicas (PESTKA, 2010; MISHRA et al., 2014). Dois grandes surtos de gastroenterite humana no Japão e na Coreia foram associados a alimentos contaminados com *Fusarium*, onde os principais sintomas foram náusea, diarreia e vômito, apontando DON como o possível agente causador. Em um relato semelhante na China, surtos de gastroenterite entre 1984 e 1991 foram associados com DON e outros cereais infectados com tricotecenos, afetando cerca de 130000 pessoas. Também na Índia, dezenas de milhares de indivíduos consumindo produtos de trigo mofados devido à exposição à chuva

onde foi relatada a presença de DON em níveis na faixa de 0,34 - 8,4 mg/kg, sofreram de gastroenterite grave (MISHRA et al., 2014).

Figura 3 – Estrutura química do deoxinivalenol:




Fonte: BENNETT;KLICH (2003)

Os principais efeitos tóxicos desencadeados por DON em nível celular devem-se ao fato de que esta micotoxina se liga ao ribossomo e causa estresse ribossômico, levando a ativação de proteíno-quinases ativadas por mitógenos (MAPK) entre outras quinases celulares, levando a inibição da síntese proteica e de ácidos nucleicos, interrupção no ciclo celular e apoptose (SHIFRIN; ANDERSON, 1999; WANG et al., 2014). DON também demonstrou interromper a sinalização celular, diferenciação, crescimento e síntese macromolecular, que está associada com efeitos de amplo espectro, como homeostase gastrointestinal, crescimento, função neuroendócrina e imunidade (PESTKA, 2010).

Além disso, vários estudos vêm indicando o estresse oxidativo como mecanismo de citotoxicidade e apoptose induzido por DON (MA; GUO, 2008; PESTKA, 2010; MISHRA et al., 2014). Foram relatados danos às membrana celulares, cromossomos e ao DNA, aumento na peroxidação lipídica, formação de espécies reativas de oxigênio e indução de apoptose e genotoxicidade (FRANKIC et al., 2006; YANG et al., 2014; DA SILVA et al., 2018). Estudos mais recentes também têm demonstrado que a exposição a DON altera a composição da microbiota intestinal, causando impactos na síntese e no metabolismo de substâncias,

desencadeando consequentes alterações metabólicas na síntese de fosfolipídios, degradação de açúcares e polissacarídios, degradação do benzoato, entre outras, bem como inflamação intestinal causada pelo desequilíbrio da microbiota (PENG et al., 2019; WANG et al., 2019).

### 1.1.4 Efeitos das micotoxinas em suínos

Os suínos são considerados os animais de produção mais sensíveis aos efeitos tóxicos das micotoxinas (HUSSEIN; BRASEL, 2001). Os efeitos crônicos da exposição diária desses animais à micotoxinas presentes na ração podem se manifestar de várias maneiras, de acordo com as micotoxinas às quais estes animais são expostos.

Diferentes animais, como aves, porcos e roedores expostos à dietas prolongadas contaminadas por OTA apresentam lesões bastante similares. Os animais apresentaram nefropatia associada à degeneração dos túbulos dos néfrons e fibrose intersticial renal, seguida por uma diminuição na espessura da membrana basal e hialinização glomerular (MARIN et al., 2013). Em suínos, essa nefropatia foi induzida em animais tratados com 4000, 1000 ou 200 µg de OTA por Kg (4, 1 ou 0.2 ppm). Nas doses de 1 – 4 ppm, os rins perderam sua coloração característica e foi observada necrose dentro de 3 e 4 meses do início do tratamento. Investigações ultraestruturais dos rins de animais expostos à 0,8 ppm de OTA identificaram um processo de condensação de material celular com desaparecimento de membranas e descamação contínua na parte inferior dos túbulos contorcidos proximais. Nas células, houve perda de integridade da membrana dos peroxissomos e extravasamento de seu conteúdo no citosol. A ingestão de água e excreção urinária dos animais é aumentada. Também foi observada perda de peso em animais tratados com doses maiores que 2 ppm de OTA (PFOHL-LESZKOWICZ; MANDERVILLE, 2007).

Além dos efeitos tóxicos da OTA em suínos, essa micotoxina tem forte afinidade às proteínas plasmáticas, ligando-se a elas e podendo assim se acumular na carne desses animais acarretando na rejeição de carcaças pelos abatedouros, aumentando ainda mais as perdas econômicas associadas a esta micotoxina. Além disso, a OTA pode afetar a fertilidade de matrizes e ainda ultrapassar a barreira placentária, afetando o desenvolvimento dos fetos. Foram observados casos de necrose da cauda em leitões recém-nascidos, muitas vezes associados à contaminação por OTA (IHESHIULOR, 2011).

Já em relação à $FB_{1,}$ de maneira geral, os principais sintomas de intoxicação em suínos são a formação de edema pulmonar, afetando os pulmões e coração. Também são observados danos no fígado e pâncreas, bem como supressão do sistema imune, além de diminuição no

ganho de peso desses animais quando alimentados com ração contaminada com níveis de $FB_1$ superiores a 2 ppm (IHESHIULOR, 2011). Em suínos, a intoxicação por $FB_1$ é caracterizada por danos na função pulmonar, cardiovascular e hepática (HASCHEK et al., 2001; BUREL et al., 2013). Foi observada a formação de edema pulmonar letal e hidrotórax, ou hidropesia da cavidade pleural, em suínos que receberam ração contaminada com mais de 12 ppm de $FB_1$. Outros estudos sugerem que o intestino também é afetado por fumonisinas, pois foi observado que a ingestão de baixas concentrações de $FB_1$ (6,5 – 13 ppm) aumenta a colonização intestinal por patógenos devido à redução na resposta inflamatória local e ao aumento na permeabilidade do epitélio intestinal (OSWALD et al., 2003; DEVRIENDT et al., 2009).

Em relação a DON, os suínos são a bastante suscetíveis a intoxicação, representando um grande risco de exposição devido ao grande percentual de trigo e milho em sua dieta (ROTTER et al., 1996). Nesses animais DON é absorvida rapidamente e eficientemente na porção superior do intestino delgado, especialmente no íleo (WACHE et al., 2009). Se a ingestão de DON ocorrer em concentrações suficientemente elevadas, pode provocar inflamação excessiva, danificando o epitélio intestinal (PESTKA; SMOLINSKI, 2005). Os principais sintomas de intoxicação de suínos por essa micotoxina são vômito e anorexia. A ração se torna não palatável para os animais, que rejeitam o alimento, ocasionando em diminuição no ganho de peso ou mesmo em perda de peso, aumento na incidência de doenças infecciosas e problemas digestivos. Já na maternidade, DON causa alterações na fertilidade das matrizes, além de causar uma série de problemas nos leitões, como inflamação no trato digestivo e diarreia aguda, o que acarreta em grande mortalidade (IHESHIULOR, 2011).

No Brasil, os limites para a presença de micotoxinas em alimentos são regulamentados pela Agência Nacional de Vigilância Sanitária (ANVISA). A normativa que estabelece os limites das micotoxinas $FB_1$ e OTA atualmente em vigor é a resolução RDC 7/2011, que estabelece os limites de OTA em 10 µg/Kg em produtos para alimentação e 2 µg/Kg para alimentos à base de cereais para alimentação infantil. Já os limites para cereais para posterior processamento são estabelecidos em 20 µg/Kg. Para as fumonisinas ($FB_1$ + $FB_2$) os limites estabelecidos são 200 µg/Kg para alimentos à base de milho para alimentação infantil, 1000 µg/Kg para amido e outros derivados de milho, 1500 µg/Kg para farinha de milho, creme de milho, fubá e canjica, e 5000 µg/Kg para milho em grãos para posterior processamento. Para o deoxinivalenol, os limites alimentos a base de cereais para alimentação infantil são de 200 µg/Kg, para trigo integral, farelo de trigo e de arroz, grãos de cevada o limite é de 1000 µg/Kg, e para arroz e produtos processados a base de trigo os limites são de 750 µg/Kg.

Porém não há uma regulamentação específica para os limites dessas micotoxinas em rações para animais.

## 1.2 ESTRESSE OXIDATIVO

Os radicais livres podem ser definidos como moléculas ou fragmentos moleculares contendo um ou mais elétrons desemparelhados. As espécies reativas de oxigênio (EROs) e as espécies reativas de nitrogênio (ERNs) são produzidas normalmente em concentrações fisiológicas pelo metabolismo celular e, dependendo de sua concentração, podem promover tanto resultados favoráveis quanto desfavoráveis para a célula (VALKO et al., 2006). Os radicais derivados de oxigênio representam a classe mais importante de espécies radicais geradas nos sistemas vivos. O ânion superóxido é a ERO primária e pode reagir com outras moléculas para gerar EROs secundárias (VALKO et al., 2007).

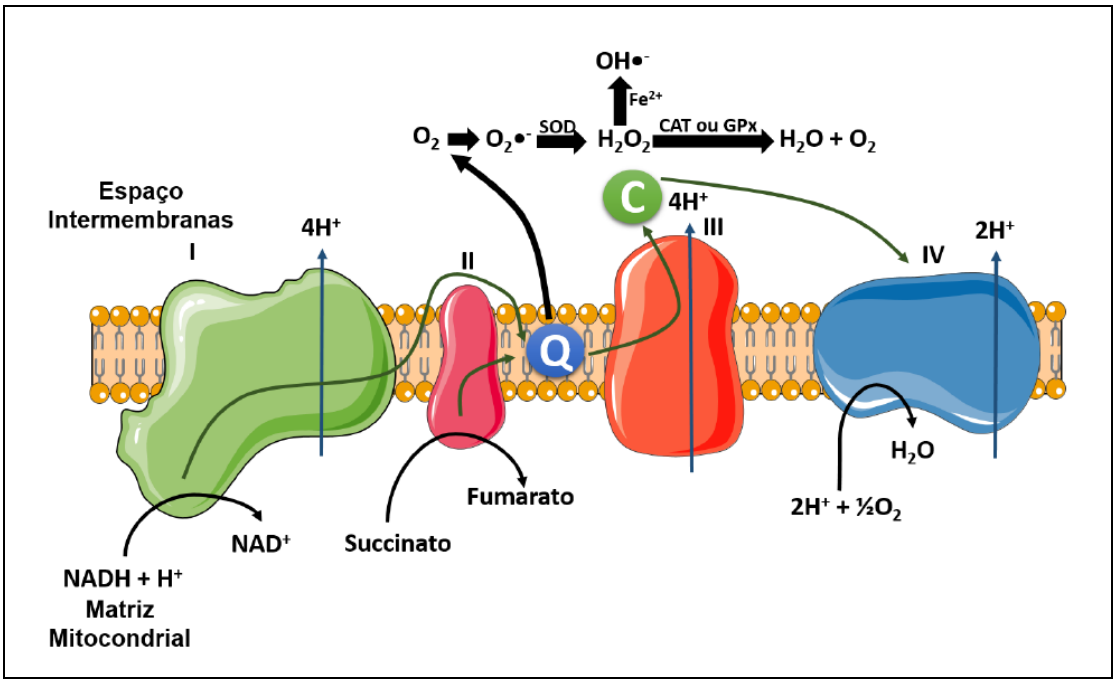
O estresse oxidativo é uma situação fisiológica caracterizada pelo desequilíbrio entre a produção de espécies reativas de oxigênio e as defesas antioxidantes do organismo. Este desequilíbrio é responsável por várias situações patológicas. Nessas situações, as EROs são produzidas de forma inespecífica como um subproduto da respiração aeróbica, sendo a mitocôndria a principal fonte endógena de EROs (FINKEL; HOLBROOK, 2000).

A mitocôndria é a organela celular de dupla membrana responsável pela produção de adenosina trifosfato (ATP) nas células eucarióticas. Em sua membrana interna, estão incrustadas as enzimas que formam a cadeia transportadora de elétrons (CTE), onde equivalentes reduzidos entregam seus elétrons provenientes de moléculas orgânicas (Figura 4). Os elétrons são transferidos entre os complexos da CTE, sendo o oxigênio molecular ($O_2$) usado como aceptor final de elétrons. A passagem de elétrons pela CTE gera o bombeamento de prótons da matriz mitocondrial para o espaço intermembranas, gerando o potencial eletroquímico necessário para a síntese de ATP no processo de fosforilação oxidativa (HATEFI, 1965).

Estimativas a partir de estudos *in vitro* afirmam que em condições normais, cerca de 0,5% dos elétrons que passam pela CTE escapam, principalmente nos complexos I e III. Nesses casos, os elétrons reagem com o $O_2$, formando o ânion superóxido, que pode reagir com proteínas e lipídios de membrana, levando a disfunções celulares e danos na membrana da célula (MURPHY, 2009). A enzima superóxido dismutase (SOD) é responsável por fazer a dismutação do ânion superóxido em peróxido de hidrogênio ($H_2O_2$), uma forma mais estável e menos reativa que o ânion superóxido. O $H_2O_2$ por sua vez, pode reagir com o íon ferroso

($Fe^{2+}$), formando o radical hidroxila e o íon férrico ($Fe^{3+}$), processo conhecido como reação de Fenton. O radical hidroxila é a ERO mais danosa às células, pois apresenta uma alta instabilidade, sendo portanto altamente reativa, causando danos oxidativos em proteínas, peroxidação lipídica, além de quebras de cadeia e modificações no DNA, não existindo nenhuma enzima que possa catalisar sua degradação (IMLAY et al., 1988).

Figura 4 – Esquema mostrando a formação de EROS que ocorre quando elétrons vazam da cadeia transportadora de elétrons




Fonte: BUENO (2015).

Os radicais livres desempenham várias funções fisiológicas quando em concentrações baixas ou moderadas, incluindo a regulação do tônus muscular, a sensibilidade à tensão de oxigênio e a regulação das funções controladas pela concentração de oxigênio, o aumento da transdução de sinal e respostas ao estresse oxidativo que asseguram a manutenção da homeostase redox. A concentração destes radicais é mantida pelo balanço entre a sua formação e sua depuração por antioxidantes (DROGE, 2002). No momento em que há uma redução no nível deste sistema de defesa antioxidante e aumento nos níveis de substâncias oxidativas, desenvolve-se o processo denominado estresse oxidativo (DROGE, 2002).

Os antioxidantes são definidos como qualquer substância que retarde, previna ou remova o dano oxidativo de uma molécula. Os organismos vivos dispõe de várias estratégias para amenizar os efeitos danosos das EROs (DAVIES, 2000). A SOD, como já mencionado, é a enzima responsável por fazer a conversão do ânion hidroxila em $H_2O_2$, que apesar de ser uma ERO, não é um radical livre, sendo então menos reativo que o ânion superóxido. Para evitar que o $H_2O_2$ reaja com o $Fe^{2+}$, as enzimas catalase (CAT) e glutationa peroxidase (GPx) fazem a quebra do $H_2O_2$ em $O_2$ e água. A CAT é uma enzima presente em grandes quantidades nos peroxissomos, enquanto a GPx degrada $H_2O_2$ com a concomitante oxidação de glutationa (GSH), sendo que algumas isoformas também são capazes de reduzir hidroperóxidos lipídicos (ARTHUR, 2000). O controle da atividade dessas enzimas pode estar relacionado com a expressão da proteína Fator Nuclear (Derivado de Eritroide 2) 2 (Nrf-2), um fator de transcrição responsável pela ativação da expressão gênica de diversas enzimas com ação antioxidante quando há uma grande quantidade de EROs nas células (ISHII et al., 2000). Além das defesas enzimáticas, sabe-se que moléculas orgânicas são capazes de sequestrar EROs de forma não-enzimática (BARTOSZ, 2010).

Devido aos vários processos patológicos causados pela produção exacerbada de EROS, várias estratégias terapêuticas estão sendo desenvolvidas como objetivo de amenizar o efeito danoso do estresse oxidativo. Essas estratégias envolvem o uso de produtos naturais e compostos sintéticos que atuam tanto no sequestro de EROs como na modulação da atividade das enzimas antioxidantes (MAXWELL, 1995).

## 1.3 COMPOSTOS FENÓLICOS

Os compostos fenólicos, também conhecidos como polifenóis, constituem um dos mais bem distribuídos grupos de metabólitos secundários de plantas, com mais de 10000 estruturas diferentes conhecidas, incluindo ácidos fenólicos, flavonoides, estilbenos e lignanas (MANACH et al., 2004). Os polifenóis são constituídos de anéis de benzeno com uma ou mais hidroxilas e apresentam uma grande variedade em suas estruturas, variando de simples fenóis à polímeros complexos, como é o caso das ligninas e suberinas (VELDERRAIN-RODRIGUEZ et al., 2014). Além disso, seus anéis aromáticos podem ser modificados por meio de hidroxilações, metilações, glicosilações, acilações e prenilação (DIXON; PAIVA, 1995; WINKEL-SHIRLEY, 2001; ANDRÉ et al., 2010).

Os compostos fenólicos têm sido relacionadas à diversos benefícios à saúde, principalmente pela sua atividade antioxidante (HEIM et al., 2002). Dietas ricas em frutas,

legumes e produtos agro-industriais são as principais fontes de obtenção de compostos fenólicos e proporcionam uma maior proteção contra danos oxidativos e doenças relacionadas (LIN et al., 2016).

### 1.3.1 Curcumina

A curcumina é um pigmento amarelo extraído do rizoma da planta *Curcuma longa* L., uma planta herbácea e perene encontrada principalmente em regiões tropicais e subtropicais, sendo originária do sudeste da Ásia e extensamente cultivada na Índia e na China. No Brasil, a *C. longa* é conhecida tradicionalmente como cúrcuma, açafrão, açafrão-da-terra, açafrão-da-Índia ou gengibre dourado. Além de ser utilizada como tempero para dar cor e sabor aos alimentos, a cúrcuma é utilizada na medicina tradicional indiana no tratamento de desordens biliares, anorexia, coriza, diabetes, doenças hepáticas, reumatismo e sinusite (SHARMA et al., 2005).

De maneira geral, a curcumina tem atraído a atenção dos pesquisadores devido às suas potenciais ações farmacológicas, incluindo propriedades antioxidantes (PIZZO et al., 2010), anti-inflamatórias (AGGARWAL; HARIKUMAR, 2009), antitumorais, promovendo apoptose em células tumorais, além de apresentar atividade anti-angiogênica (LEE et al., 2009; VALLIANOU et al., 2015), anti-leshimaniose e antifúngica (KAMINAGA et al., 2003), antibacteriana (DE et al., 2009) e antiviral (MOGHADAMTOUSI et al., 2014).

A parte de maior interesse da cúrcuma é o rizoma, pois é a partir da moagem dos rizomas secos que se obtém o pó, que é muito utilizado na indústria alimentícia como corante, aromatizante e tempero, sendo um dos principais constituintes do *curry* (ANTUNES et al., 2000). A cúrcuma pode conter de 3 a 15% de curcuminoides e de 1,5 a 5% de óleos essenciais (LI et al., 2011). O cultivo da cúrcuma vem ganhando espaço no mercado mundial como solução na substituição de corantes sintéticos, além de ser utilizado também pelas suas propriedades medicinais e farmacológicas (SCARTEZZINI; SPERONI, 2000).

A atividade biológica da cúrcuma vem sendo atribuída a presença de curcumina, um polifenol lipossolúvel que apresenta em sua estrutura dois anéis de metoxifenol unidos por uma β-dicetona. Os grupos fenólicos e a β-dicetona são característicos de compostos antioxidantes, o que torna essas estruturas responsáveis pela atividade antioxidante da curcumina (ARAUJO; LEON, 2001; XIAO et al., 2010; SINGH et al., 2011).

A curcumina [(E,E)-1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6- heptadiene-3,5-ione] é extraída da *C. longa* juntamente com a demetoxi-curcumina e a bisdemetoxi-curcumina

(BHAWANA et al., 2011). As três principais substâncias podem representar 90% do total de curcuminoides. A curcumina é um pó de coloração amarelada, cristalino, de fórmula molecular $C_{21}H_{20}O_6$ e peso molecular de 368,38 g/mol. Além disso, é praticamente insolúvel em água (LI et al., 2011).

A atividade antioxidante comparativa da curcumina, desmetoxi-curcumina e bisdesmetoxi-curcumina, tem sido estudada em modelos *in vitro*, demonstrando que a desmetoxi-curcumina e a bisdesmetoxi-curcumina também são bons antioxidantes, juntamente com a curcumina (JAYAPRAKASHA et al., 2006). A curcumina apresentou atividade antioxidante eficaz em diferentes ensaios *in vitro* quando em comparação com os compostos antioxidantes convencionais. Pode ser usada para minimizar ou prevenir oxidação lipídica de produtos farmacêuticos, retardando a formação de produtos de oxidação tóxicos, mantendo a qualidade nutricional e prolongando a vida de prateleira (AK; GULCIN, 2008).

Já em relação ao sistema imunológico, a curcumina mostrou-se um potente agente imunomodulador celular que pode ativar linfócitos T e B, macrófagos, neutrófilos, células *natural killers* (NK) e células dendríticas (DCs). Além disso, a curcumina tem funções de modular a resposta de citocinas e quimiocinas, reduzindo a produção de fator de necrose tumoral (TNF) e a produção de interleucinas (IL), e inativa a transcrição de fator NF-κβ. A ingestão de baixas doses de curcumina também tem mostrado efeito benéfico em doenças autoimunes como Alzheimer, diabetes, esclerose múltipla, alergias, asma e artrite reumatoide. Sua aplicação tópica em gel de curcumina 1% demonstrou redução na densidade de células T $CD8^+$, mostrando efetividade no tratamento de psoríase (JAGETIA; AGGARWAL, 2007).

### 1.3.2 Silimarina

A silimarina é um composto fenólico extraído dos frutos e sementes da planta *Silybum marianum*, (L.) Gaertn. Essa planta pertence à família Asteraceae, cresce em solos rochosos podendo atingir até um metro e meio de altura, apresentando flor de cor arroxeada e folhas com nervuras de aspecto leitoso, o que confere o nome vulgar da planta “Milk thistle”. O fruto tem de 5-7 mm de comprimento, 2-3 mm de largura e 1,5 mm de espessura, de cor castanha brilhante (KROLL et al., 2007). *S. marianum* é nativo do sul da Europa, sul da Rússia, Ásia Menor e norte da África e se adaptou ao norte e sul da América, bem como no sul da Austrália.

Os principais componentes da silimarina isolados e caracterizados estruturalmente são a silibina, isosilibina, silicristina e silidianina (SONNENBICHLER et al., 1999). No entanto,

Johnson e colaboradores (2002) descrevem o produto como uma mistura de sete isômeros denominados taxofolina, silicristina, silidianina, silibina A, silibina B, isosilibina A e isosilibina B (JOHNSON et al., 2002). As sementes de *S. marianum* também contêm betaína, que apresenta comprovado efeito hepatoprotetor (LUPER, 1998). Além disso, a silimarina é constituída de ácidos graxos essenciais o que pode contribuir para sua atividade antiinflamatória (LUPER, 1998).

A silimarina apresenta diversas atividades descritas na literatura, como hepatoprotetora e regeneradora (CROCENZI; ROMA, 2006), antioxidante (WELLINGTON; JARVIS, 2001; SANGEETHA et al., 2010), antifibrótica (FLORA et al., 1998; JIA et al., 2001; LIN et al., 2008) e antiinflamatória (SCHUMANN et al., 2003), tratando-se, portanto, de um fitoterápico de uso promissor. A atividade hepatoprotetora da silimarina é devida, principalmente, à captura de radicais livres produzidos por eventos diversos nas células, evitando os danos promovidos pelo estresse oxidativo e efeitos antiinflamatórios (AL-RASHEED et al., 2016). Dessa forma, a silimarina apresenta atividade quimiopreventiva e anticancerígena através da captura de radicais livres (DORAI; AGGARWAL, 2004), modulação de mitógenos, sinalizadores de ciclo celular e sobre proteínas envolvidas na apoptose, modulando o desequilíbrio entre a sobrevivência celular e a apoptose (RAMASAMY; AGARWAL, 2008) com consequente atividade antineoplásica (SINGH; AGARWAL, 2002; TYAGI et al., 2009).

Além disso, o uso da silimarina já foi proposto na terapia adjuvante do câncer, protegendo os tecidos do estresse oxidativo gerado pelos quimioterápicos e reduzindo a hepatotoxicidade (INVERNIZZI et al., 1993; SCAMBIA et al., 1996), bem como nova possibilidade para tratamento do diabetes mellitus por aumentar os níveis de insulina em hiperglicemias e recuperar a função pancreática (SOTO et al., 2004). Além disso, a silimarina pode ter um longo tempo de ação no fígado (HE et al., 2002) e não é hepatotóxica baseadas em observações clínicas, já que apresenta valores muito altos de dose letal 50 - LD50 (FLORA et al., 1998).

### 1.3.3 Efeitos de compostos fenólicos e antioxidantes sobre os danos causados pelas micotoxinas

Diversos estudos têm demonstrado que compostos fenólicos e outros antioxidantes têm a capacidade de proteger e até mesmo neutralizar os danos causados por várias substâncias tóxicas. Com as micotoxinas não é diferente. Estudos *in vitro* mostraram que o

resveratrol, um polifenol encontrado na casca de uvas danificadas e outras frutas (ATHAR et al., 2007), é capaz de prevenir o dano causado pela OTA em células renais devido à sua atividade antioxidante, uma vez que essa micotoxina parece esgotar as defesas antioxidantes das células e dessa forma produzir danos às membranas, proteínas e ao DNA das células-alvo (RAGHUBEER et al., 2015). Outro estudo com o resveratrol mostrou que este polifenol também é capaz de proteger células renais contra a toxicidade induzida pela zearalenona, uma micotoxina estrogênica não-esteroidal, também devido à sua atividade antioxidante (SANG et al., 2016).

Além disso, foi demonstrado que a curcumina é capaz de neutralizar o estresse oxidativo causado pela zearalenona em células da granulosa de suínos, em um estudo *in vitro*, a partir da análise da expressão gênica de enzimas que desempenham um papel crucial nos mecanismos oxidativos celulares e da detecção de radicais livres nas células expostas à micotoxina e tratadas com a curcumina (QIN et al., 2015). Além disso, curcumina foi capaz de diminuir a hepatotoxicidade e hepatocarcinogenicidade induzida pela micotoxina aflatoxina B1 (AFB1) em ratos, por meio da modulação de enzimas antioxidantes de enzimas importantes no metabolismo de drogas (EL-BAHR, 2015; POAPOLATHEP et al., 2015). Outro estudo, comparando o efeito da curcumina e do resveratrol frente à toxicidade induzida pela AFB1 em ratos, mostrou por meio de análises de biomarcadores oxidativos e de exames histopatológicos, que a curcumina foi capaz de atenuar os danos hepáticos causados pela AFB1 (EL-AGAMY, 2010).

Em relação à silimarina, estudos *in vivo* demonstram a capacidade desse polifenol em evitar danos hepáticos causados por micotoxinas em aves e roedores. Em camundongos BALB/c que receberam ração contaminada com fumonisina $B_1$ foi demonstrado que a silimarina foi capaz de reverter danos hepáticos causados pela micotoxina (HE et al., 2004; SOZMEN et al., 2014). Em ratos, a silimarina foi capaz de proteger as células hepáticas contra os danos causados pela AFB1, melhorando o transporte de membranas, a cadeia transportadora de elétrons, transcrição gênica e estresse oxidativo (RASTOGI et al., 2000). Da mesma forma, em aves, a silimarina mostrou-se eficiente na proteção contra danos hepáticos induzidos por AFB1 (GRIZZLE et al., 2009) e, juntamente com vitamina E, contra efeitos imunotóxicos causados pela OTA (KHATOON et al., 2013).

# 2 OBJETIVOS

## 2.1 OBJETIVO GERAL

Avaliar o potencial da curcumina e da silimarina na prevenção da toxicidade aguda causada por micotoxinas.

## 2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a toxicidade da ocratoxina A em células mononucleares do sangue periférico de suínos e células de rim suíno da linhagem PK-15 através da avaliação da viabilidade celular, estresse oxidativo e atividade mitocondrial;

- Avaliar o efeito protetor do pré-tratamento de curcumina e silimarina sobre células renais da linhagem PK-15 de suínos *in vitro* sobre a toxicidade induzida por ocratoxina A, fumonisina $B_1$ e deoxinivalenol, por meio da avaliação de viabilidade celular, estresse oxidativo e apoptose;

- Investigar o efeito do pré-tratamento com curcumina e silimarina na prevenção da toxicidade aguda causada por fumonisina $B_1$ e ocratoxina A sobre o estresse oxidativo hepático e renal, bem como sobre biomarcadores sorológicos de função hepática e renal em camundongos *in vivo*.

## 3 PROTOCOLO EXPERIMENTAL I

OCHRATOXIN A EXPOSURE INDUCES OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION IN PORCINE KIDNEY PK-15 CELLS AND IN PORCINE PBMCS *IN VITRO*

Manuscrito submetido ao periódico: *Toxicology and Applied Pharmacology*.

[1]Comprovante de submissão no anexo A.

[2]Certificado de aprovação junto ao CEUA no anexo B.

# Ochratoxin A exposure induces oxidative stress and mitochondrial dysfunction in porcine kidney PK-15 cells and in porcine PBMCs *in vitro*

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**Abstract**

Ochratoxin A (OTA) is a worldwide distributed food and animal feed contaminant known to induce a series of toxic effects in human and animals including nephropathy with progressive renal failure. This study investigated the toxic effects of OTA in porcine kidney PK-15 cell line and fresh porcine PBMCs *in vitro*, using a range of concentrations of OTA, varying from 0.5 to 20 µM along with an untreated control. The following techniques were used: cellular viability was quantified using MTT assay and LDH activity, reactive species were measured using the DCFH-DA assay, lipid damage was accessed via TBARS assay, the activity of the antioxidant enzymes SOD and CAT was quantified and oxygen consumption rates of PK-15 cells exposed to OTA for 24 h were evaluated polarographically in a high-resolution respirometry (HRR) apparatus. The results showed that OTA caused loss in cell viability and an increase in plasma membrane permeability in the concentration of 5 µM and above after 24 h ($P < 0.05$); an increase in oxidative stress indicated by reactive species formation ($P < 0.01$) and lipid peroxidation ($P < 0.001$), besides an imbalance in SOD activity ($P < 0,05$) and a decrease in CAT activity ($P < 0.001$). The results also showed similar effects in both cell types as consequence of the exposure to OTA. Moreover, the oxygen consumption rates in PK-15 cells exposed to OTA were significant altered in comparison to the control, with an inhibition of the electron transport system. The findings of this study demonstrate that OTA exerts its toxicity through oxidative stress and inhibition of enzymes of the endogenous antioxidant system, besides changes in mitochondrial functioning.

**Key-words:** Ochratoxin A; PK-15 cell line; PBMCs; Oxidative stress; Toxicity.

## Introduction

Mycotoxins are toxic secondary metabolites produced by fungus which have caused worldwide concern regarding food and feed safety because of their global distribution and harmful effects (Burel *et al.*, 2013). Ochratoxin A was initially described in South Africa in 1965 (van der Merwe *et al.*, 1965). This mycotoxin is mainly produced by *Aspergillus ochraceus* and *Penicillium verrucosum* being globally distributed. OTA is a highly abundant food and animal feed contaminant and is frequently detected in grains, cereals and cereal products, and also in fruit, coffee, spices, nuts, among other food (Heussner and Bingle, 2015). Besides, since OTA has an stable structure, it is not much affected by high temperatures used in food processing (Bullerman and Bianchini, 2007).

An important problem regarding mycotoxins is livestock feed contamination. Pigs are considered to be the farm animals which are the most affected and most sensitive to mycotoxins in general (Hussein and Brasel, 2001; Burel *et al.*, 2013). In pigs, it has been already shown that OTA can affect the growth and reduce the body weight gain in 24%, up to 52% when combined with other mycotoxins such as aflatoxins (Hussein and Brasel, 2001). Additional symptoms of OTA toxicity in swine include anorexia, faintness, uncoordinated movement, and increased water intake and urination (Hussein and Brasel, 2001). OTA is also considered a causative agent in the development of nephropathy in several types of farm animals (O'Brien and Dietrich, 2005) and causes destruction of the renal tubular epithelium resulting in progressive renal failure.

In humans this disease is particularly prominent in the Balkan regions, such as Bulgaria and Romania, and it is known as Balkan endemic nephropathy (Raghubeer *et al.*, 2015). Under experimental conditions, OTA has shown a diverse range of toxicological

effects, including nephrotoxicity, teratogenicity, immunotoxicity, neurotoxicity and hepatotoxicity (Wangikar *et al.*, 2005; Gagliano *et al.*, 2006; Pfohl-Leszkowicz and Manderville, 2007; Sava *et al.*, 2007; Zhang *et al.*, 2009; Babayan *et al.*, 2020).

The toxic effects of OTA are related to a number of mechanisms, but the one that stands out is the imbalance of the cellular oxidative stress which results in an increase in reactive oxygen species (ROS) (Schaaf *et al.*, 2002). In normal conditions, cells produce reactive species in a balanced way, but once the cellular redox balance is disrupted it results in oxidative damage to the cells, which can lead to apoptosis (Jones, 2008). However, the mechanisms underlying the oxidative stress induced by OTA are not completely understood. Therefore, in this study we investigated OTA toxicity and its effects on oxidative stress biomarkers in porcine kidney PK-15 cell line and porcine peripheral blood mononuclear cells (PBMCs).

## Materials and methods

### *Obtaining cells and culture conditions*

Porcine kidney PK-15 cell line (ATCC) were cultured in Minimum Essential Medium (MEM – Sigma-Aldrich, St Louis, USA) containing 5% Fetal Bovine Serum (FBS – Vitrocell, Campinas, Brazil) and 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St Louis, USA). Prior to experiments, Peripheral Blood Mononuclear Cells (PBMCs) were isolated from porcine peripheral blood by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, St Louis, USA). Briefly, after centrifugation, the cell pellets were washed with Phosphate Buffered Saline (PBS) pH 7.4 and resuspended in RPMI 1460 medium (Sigma–Aldrich, St Louis, USA) supplemented with 10% Fetal Bovine Serum

and 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured in an incubator at 37 ºC in a 5% humidified $CO_2$ atmosphere. The blood samples were collected from a 3-year-old adult male pig, created and maintained in an animal facility for pigs at Universidade Federal de Santa Maria. All blood samples were collected from the same animal. All protocols were approved by the Animal Care and Use Committee from Universidade Federal de Santa Maria (CEUA/UFSM) (protocol number 1185101117). This study was carried out in accordance with international recommendations for animal care.

*Cell viability assay by MTT reduction and LDH activity*

The viability of PK-15 cells and PBMCs was evaluated based on the method described by Mosmann (1983) with slight modification. The cells were plated in 96-well microplates at a cell density of $5 \times 10^4$ cells/mL for PK-15 cells and $1 \times 10^6$ cells/mL for PBMCs. PK-15 cells were incubated overnight to allow cells to attach to the plate. Afterwards, cells were treated with different concentrations of OTA (Sigma-Aldrich, St Louis, USA), varying from 0.5 to 20 µM along with an untreated control (CTL). After treatments, cells were incubated for 24 and 72 h at 37 ºC under a 5% humidified $CO_2$ atmosphere. After the incubation time 20 µL of the supernatant were collected and stored to perform LDH activity assay and 20 µL of a 5 mg/mL MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St Louis, USA) solution were added into each well. After 2 h incubation time the plates were centrifuged at 1500 rpm for 5 min at room temperature. Furthermore, after centrifugation the supernatant was carefully discarded and 200 µL of dimethyl sulfoxide (DMSO) were added into each well. The absorbance was measured at 590 nm in a microplate reader UV/Visible spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The viability of CTL group was calculated from the mean absorbance and represented as 100%.

The release of cytosolic enzyme LDH in the culture medium from cells was used as parameter of plasma membrane damage. The nicotinamide adenine dinucleotide (NADH) produced by enzyme after oxidation of lactate to pyruvate was colorimetrically quantified by standard spectroscopy using a commercial LDH activity kit (Labtest, Minas Gerais, Brazil), following the protocol recommended by the manufacturer. The results were expressed as Units of LDH per liter of sample (U/L).

*Determination of reactive species production*

Reactive species production was evaluated according to the method described by Esposti (2002) with slight modifications. PK-15 cells were seeded into a 96-well plate ($5 \times 10^4$ cells/mL) and incubated overnight to allow cells adhesion to the plate and then treated with different concentrations of OTA. Likewise, PBMCs were seeded into a 96-well plate ($1 \times 10^6$ cells/mL) and treated with different concentrations of OTA. The plates were then incubated for 24 hours at 37 ºC in a 5% humidified $CO_2$ atmosphere. After the incubation time, 100 µL of supernatant was collected, transferred to a black 96-wells microplate containing 130 µL of Tris HCl Buffer (1mM) and 20 µL of 2,7-Dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich, St Louis, USA) solution were added into each well (the final concentration of DCFH-DA in each well was 10 µM). The plates were incubated in the dark for 1 hour and subsequently read on a SpectraMax i3x multi-mode microplate reader. The fluorescence of oxidative derivates (DCF) was read at 488 nm of excitation and 525 nm of emission. The results were expressed as percentage of fluorescence emitted by the samples compared to the CTL group, represented as 100%.

*Lipid peroxidation: TBARS assay*

The thiobarbituric acid reaction was performed as described by Ohkawa *et al.* (1979) with minor modifications. Both PK-15 cells and porcine PBMCs were plated, treated with different concentrations of OTA and incubated for 24 hours as previously described. After the incubation time the cells and the supernatant were collected to perform the assay. In order to perform the reaction, 100 µL of acetic acid 20% (pH 3.0), 150 µL of TBA 0.8% (pH 3.0), 50 µL SDS 8.1% and 100 µL of the samples were added to the reaction tubes and incubated in a hot bath at 95 ºC for 1 hour and subsequently transferred to a 96-wells plate and read on a SpectraMax i3x multi-mode microplate reader at 532 nm of absorbance.

*Superoxide dismutase (SOD) and catalase (CAT) activity*

Total SOD activity in PK-15 cells and porcine PBMCs was measured by an indirect assay based on the competitive reaction between SOD and nitroblue tetrazolium chloride. The rate of increase in the absorbance at 560 nm indicates the reduction of nitroblue tetrazolium chloride to blue formazan by superoxide, which are generated by the xanthine/xanthine oxidase system. The enzymatic reaction was initiated by adding 20 µL of xanthine oxidase to the reaction tube. One unit of activity was defined as the amount of protein necessary to achieve half-maximal inhibition of the nitroblue tetrazolium chloride reaction, and the activity was expressed as units per mg of protein (Unfer *et al.*, 2015).

CAT enzyme activity in cells exposed to OTA was measured with the method of Aebi (1984) with slight modifications. For the determination of the CAT activity, a 10 µL aliquot of cells was added to a cuvette, and spectrophotometric determination was started by the addition of 35 µL of freshly prepared 0.3 mol/L $H_2O_2$ solution in potassium phosphate buffer

(50 mM, pH 7.0) to give a final volume of 1 mL. The rate of the $H_2O_2$ reaction was monitored at 240 nm for 2 min at room temperature. The CAT activity was calculated using the molar extinction coefficient (0.0436 $cm^2$/µmol), and the results were expressed as µmol/mg of protein.

*High resolution oxygen respirometry*

Oxygen consumption rates of PK-15 cells exposed to 10 µM of OTA for 24 h were evaluated polarographically in a high-resolution respirometry (HRR) apparatus equipped with a fluorescence detection module (Oroboros Oxygraph-O2K, Oroboros Innsbruck, Austria) in a medium containing KCl buffer (80 mM KCl, 10 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) at 37 °C, following the method described by Sangle *et al.* (2010), with some modifications. The number of PK-15 cells added in each chamber, control or treated, was 6 x $10^5$ cells/mL. The protocol consisted of a sequential titration of multiple substrates, uncouplers and inhibitors (SUIT protocol) (Pesta and Gnaiger, 2012). Thus, after signal stabilization, the experimental protocol was performed by permeabilization of cells with 15 µg digitonin and sequential addition of 5 mM pyruvate /5 mM malate/ 10 mM glutamate, 4 mM ADP, 10 mM succinate, 2.5 µM oligomycin, a titration with carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), 100 nM by 100 nM, until the maximal respiratory capacity to be reached, 0.5 µM rotenone, 5 mM malonate and 2.5 µM antimycin A. DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition and analysis.

*Statistical analysis*

All experiments were performed in three different days, in triplicates each day. The results were evaluated statistically by analysis of variance (ANOVA) with Dunnet's test for the *in vitro* analysis using the Graph Pad Prism version 6.0 software and were expressed as the mean ± SEM. The level of statistical significance was defined as $P < 0.05$.

## Results

*Inhibition of cell viability: MTT and LDH assay*

The cytotoxic effect of OTA on PK-15 cells and porcine PBMCs measured by MTT assay after 24 and 72 h incubation is shown in Fig 1. Cells were treated with OTA in concentrations ranging from 0.5 – 20 µM and a significant decrease of cell viability was observed in PK-15 cells treated with 5 µM and higher concentrations of OTA in 24 h (Fig 1A) and 1 µM and higher concentrations in 72 h (Fig 1B). PBMCs showed significant decrease in cell viability with 5 µM and higher concentrations in 24 h (Fig 1C) and 2.5 µM and higher concentrations of OTA after 72 h of incubation (Fig 1D).

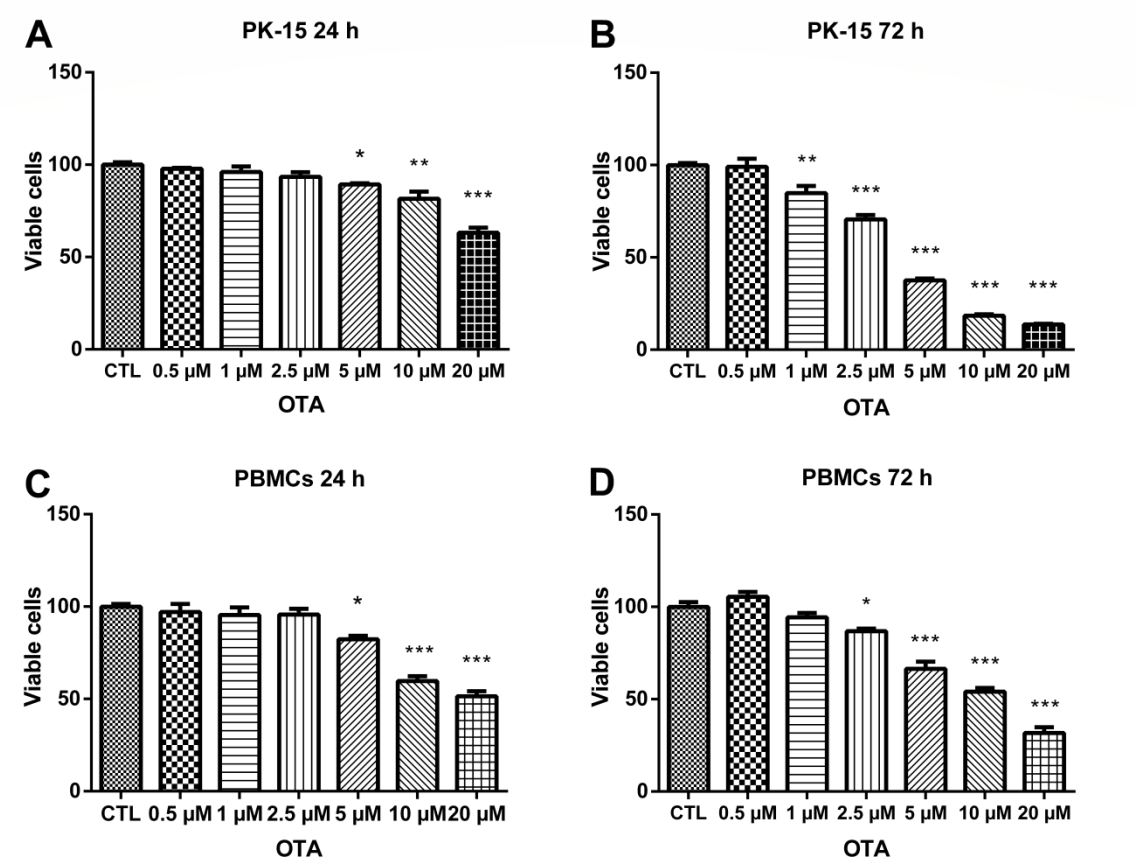


**Figure 1:** Viability of PK-15 cells exposed to different concentrations of ochratoxin A (OTA) by MTT reduction after 24 (A) and 72 (B) hours of incubation, and porcine PBMCs after 24 (C) and 72 (D) hours of incubation. Results are expressed as % of the control group (CTL) and represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicate statistical difference from control by One-way ANOVA followed by Dunnett test.

Moreover, LDH leaking was increased in PK-15 cells and also in PBMCs exposed to different concentrations of OTA (Fig 2). In PK-15 cells, LDH leaking was significantly increased at 10 µM and 20 µM after 24 h (Fig 1B), and in concentrations from 2.5 µM to 20 µM after 72 h of incubation (Fig 2B). In porcine PBMCs, LDH leaking was also increased at 10 µM and 20 µM after 24 h of incubation (Fig 2C), and at the same concentrations after 72 h (Fig 2D).

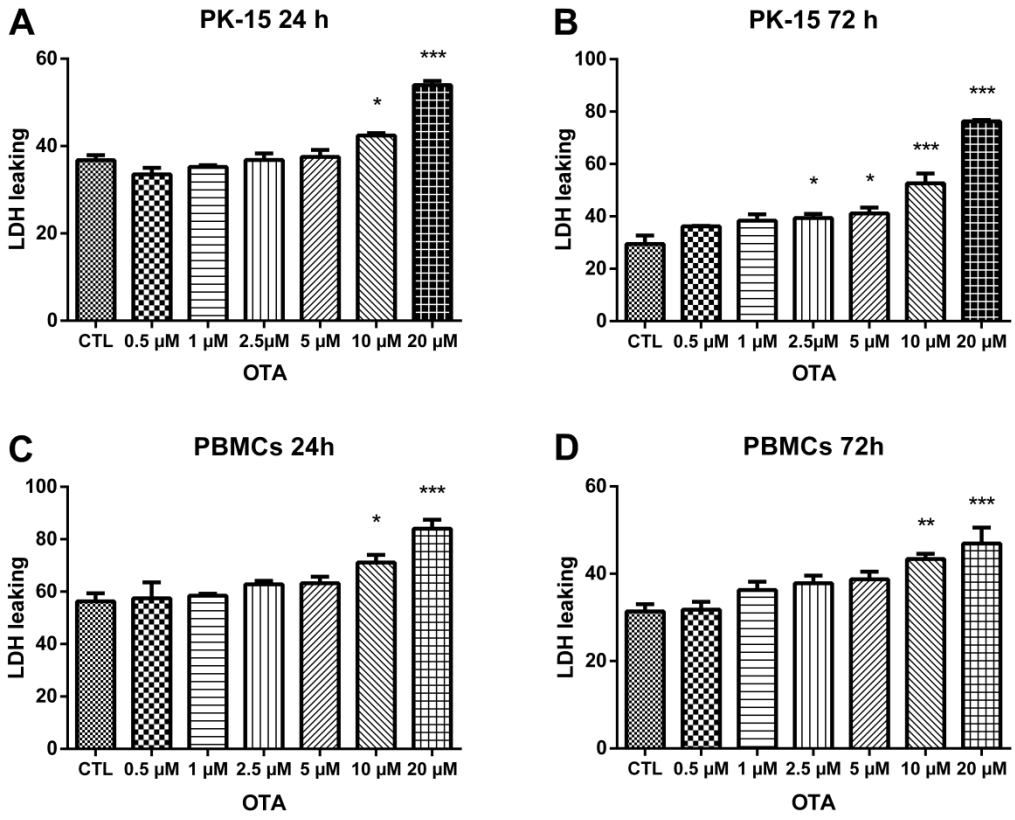


**Figure 2**: LDH activity detected in the supernatant of PK-15 cells exposed to different concentrations of ochratoxin A (OTA) after 24 (A) and 72 (B) hours of incubation, and porcine PBMCs after 24 (C) and 72 (D) hours of incubation. Results are expressed as U/L of enzyme detected in the culture medium. Data represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicate statistical difference from control (CTL) by One-way ANOVA followed by Dunnett test.

*Reactive species production*

Reactive species were evaluated by DCFH-DA assay. Based on the results of the cell viability assays of PK-15 cells exposed to OTA at two different incubation times at concentrations varying from 0.5 to 20 µM, we decided to evaluate the oxidative markers only in the time of 24 hours of incubation and eliminated the highest concentration of the mycotoxin, since in 72h a very high toxicity was observed in that concentration. Reactive species levels in porcine PK-15 cells exposed to OTA were significantly increased at the

concentration of 10 µM after 24 h of incubation (Fig 3). At 10 µM, OTA increased the fluorescence of PBMCs in about 30%, while in PK-15 cells the fluorescence was increased by 20% at the same concentration of OTA when compared to the CTL group.

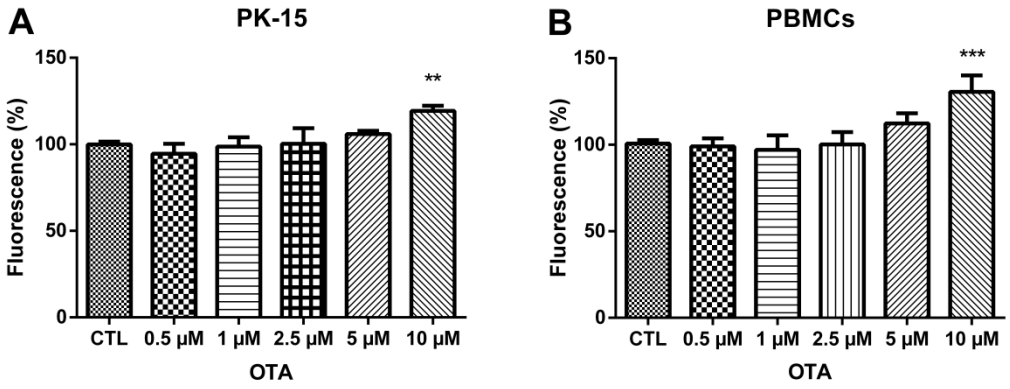


**Figure 3:** Reactive species generation of PK-15 (A) and porcine PBMCs (B) exposed to different concentrations of ochratoxin A (OTA) after 24 hours of incubation. Results are expressed as % of the control group (CTL) and represent the mean ± SD. **P < 0.01, ***P < 0.001 indicate statistical difference from control by One-way ANOVA followed by Dunnett test.

*Lipid peroxidation*

The production of malondialdehyde (MDA), a secondary metabolite formed in lipid peroxidation, was evaluated by TBARS assay. In PK-15 cells, we observed an increase in MDA formation indicating that OTA enhanced lipid peroxidation in at 5 and 10 µM. Also, OTA increased MDA formation in about 35% in cells exposed to 5 µM and 60% in cells exposed to 10 µM of OTA after 24 h of incubation (Fig 4A). In porcine PBMCs, OTA significantly increased MDA formation at 2.5 µM and higher concentrations. Moreover, at 10 µM OTA, MDA formation was about 35% higher than the CTL group after 24 h of exposure (Fig 4B).

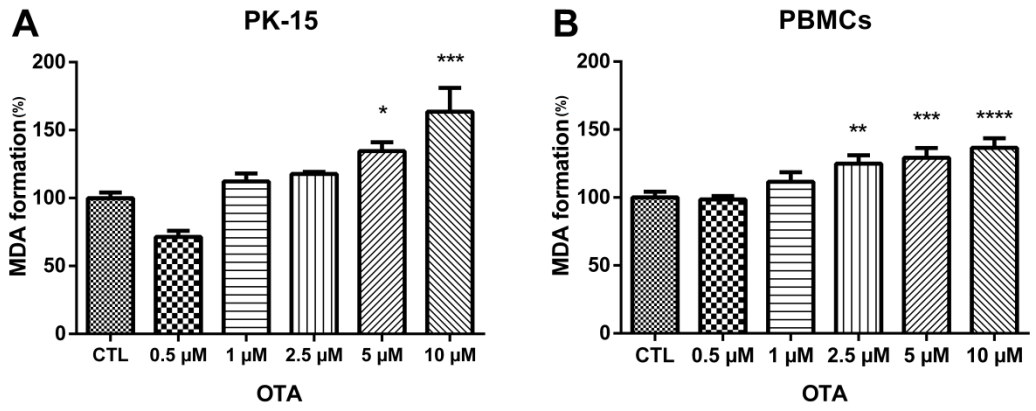


**Figure 4:** MDA levels of PK-15 (A) and porcine PBMCs (B) exposed to different concentrations of ochratoxin A (OTA) after 24 hours of incubation. Results are expressed as % of the control group (CTL) and represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicate statistical difference from control by One-way ANOVA followed by Dunnett test.

*High resolution oxygen respirometry*

OXPHOS CI-Linked and OXPHOS CI&CII-Linked states were significantly enhanced by ochratoxin A treatment in PK-15 cells (Fig. 5B). This effect was observed after addition of pyruvate/malate/glutamate and ADP, but before the addition of succinate, following the SUIT protocol (Fig. 5A). However, ETS CI&CII-Linked and ETS CI-Linked were significantly inhibited by OTA, in comparison to control (Fig. 5B). This data was evaluated after addition of the uncoupler FCCP to the running (Fig. 5A). Routine respiration, PMG, OXPHOS CII-Linked states were not changed by OTA (Fig. 5B).

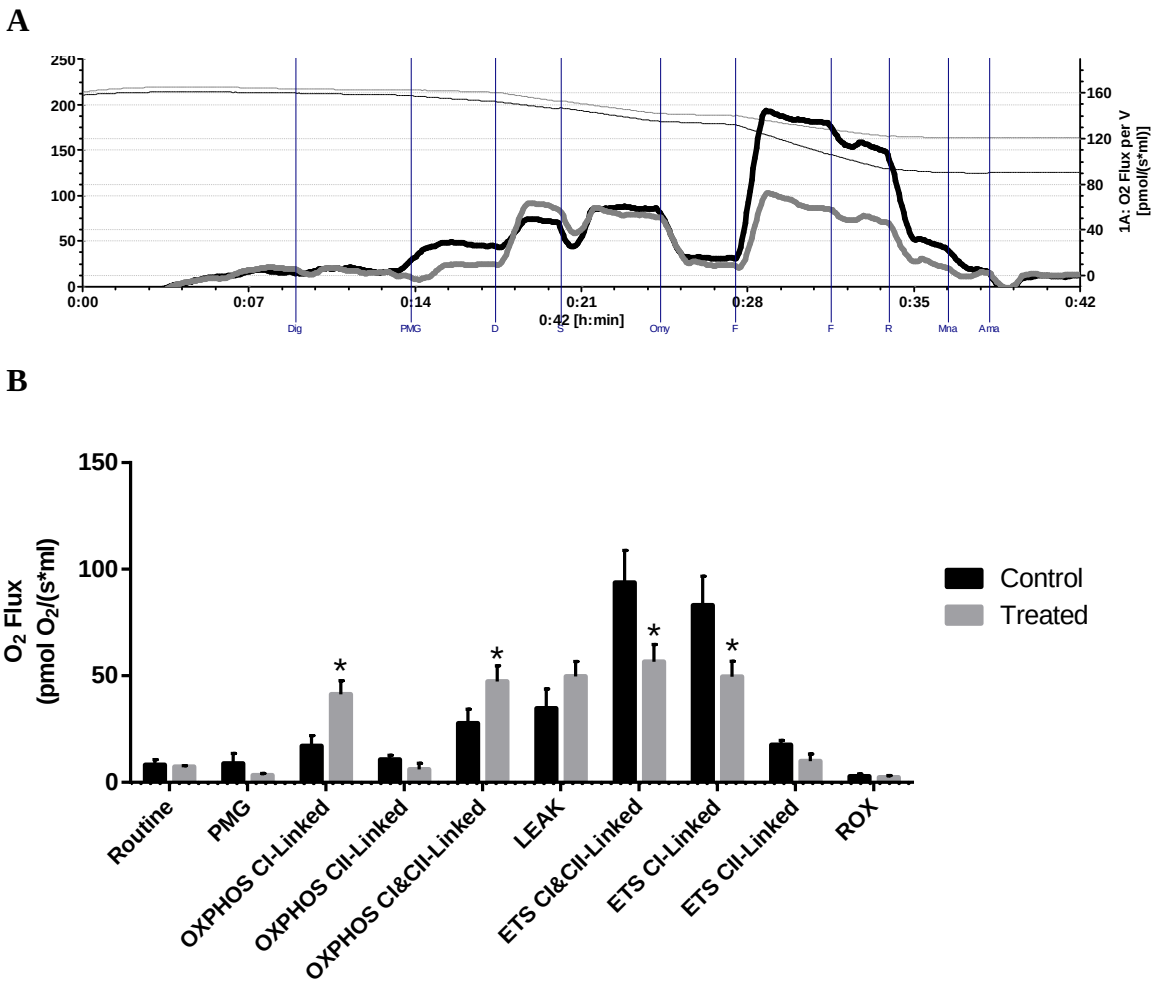


**Figure 5:** Effect of treatment of PK-15 cells with ochratoxin by high-resolution respirometry (HRR). Legend: Dig: digitonin; PMG: pyruvate/malate/glutamate; D: ADP; S: succinate; Omy: oligomycin; F: FCCP; R: rotenone; Mna: malonate; Ama: antimycin A; OXPHOS: oxidative phosphorylation; CI: complex I; CII: complex II; ETS: electron transport system. (A) Oxygen consumption (thin line) and oxygen flux (thick line) rate of SUIT protocol for Oroboros system. Thin line represents oxygen concentration inside to Oroboros chambers and thick line represents electron transfer related to oxygen flux response to substrate, uncoupler or inhibitors addition. (B) SUIT protocol results: Routine is the respiration state without any substrates. Pyruvate, malate and glutamate were used to evaluate oxygen flux without phosphorylation (PMG). OXPHOS represents coupled states dependent on different mitochondrial substrates: pyruvate, malate, glutamate (OXPHOS CI-Linked) and succinate (OXPHOS CII-Linked and OXPHOS CI&CII-Linked) in presence of saturated ADP concentrations. LEAK is the state related to ATP-synthase inhibition by oligomycin. ETS represents maximum oxygen flux by addition of the uncoupler FCCP (ETS CI&CII-Linked). In sequence, after addition of rotenone, it is demonstrated the oxygen flux related to complex I (ETS CI-Linked). The oxygen flux related to complex II is measured after addition of malonate (ETS CI-Linked), and the oxygen residual flux (ROX) is observed after complex III inhibition with antimycin A. * $P < 0.05$ compared with the control group, by unpaired test t.

*SOD and CAT activity*

As showed in Fig. 6, total SOD activity responded differently as OTA concentrations increased. In a first moment, SOD activity significantly increased when compared to the CTL group in the lower concentrations – 0.5 µM for PK-15 cells and 1 µM for porcine PBMCs. In the intermediary concentrations of OTA, SOD activity did not differ from the CTL group in both cell types. However, at 5 and 10 µM of OTA in PK-15 cells and 10 µM in PBMCs, total SOD activity significantly decreased when compared to the CTL group. CAT activity was significantly reduced in both PK-15 cells and porcine PBMCs exposed to concentrations from 1 µM to 10 µM of OTA (Fig. 6C and 6D).

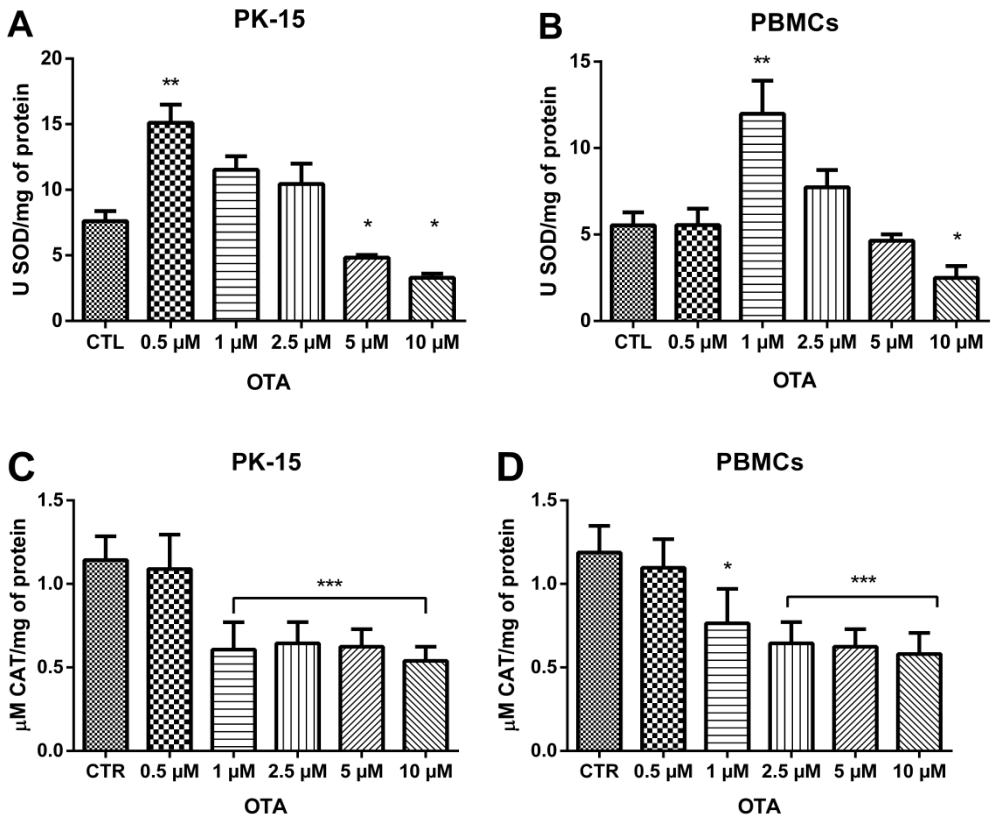


**Figure 6:** SOD activity of PK-15 (A) and porcine PBMCs (B) and CAT activity of PK-15 cells (C) and PBMCs (D) exposed to different concentrations of ochratoxin A (OTA) after 24 hours of incubation. SOD results are expressed as U/mg of protein and CAT results are expressed as µM of CAT/mg of protein and represent the mean ± SD. *P < 0.05, **P < 0.01,

***P < 0.001 indicate statistical difference from control (CTL) by One-way ANOVA followed by Dunnett test.

**Discussion**

Based on the evidence currently available, oxidative stress and ROS are believed to be the cause of toxicity induced by OTA. The mechanisms of the oxidative pathway is not yet completely understood, although this mycotoxin is known to promote pro-oxidant conditions, inducing oxidative stress, protein synthesis disruption and lipid and DNA damage in *in vivo* models (Rahimtula *et al.*, 1988; Gagliano *et al.*, 2006). In this sense, oxidative stress is caused by an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Moreover, disturbances in the normal redox status of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA (Schieber and Chandel, 2014).

Nephrotoxicity caused by OTA is mostly species-dependent, mainly due to differences in elimination half-life of OTA which varies significantly between different species. The half-life of OTA in pigs which received a single i.v. application of the mycotoxin was estimated to 150 hours (Dietrich *et al.*, 2005). In the present study we have chosen porcine cells because of the high susceptibility of pigs to OTA toxic effects and their importance in livestock production. We investigated the cytotoxic effects of OTA in two different porcine cell types, the renal epithelial PK-15 cell line and fresh PBMCs. Our results are in accordance with the oxidative hypothesis in what regards ROS formation. As seen in fig 3, reactive species increased in cells treated with OTA by DCF fluorescence in both porcine PK-15 renal cells and PBMCs. Besides, we observed an increase in MDA formation as a result of lipid peroxidation of cells treated with different concentrations of OTA, as seen in fig 4. The

enzymatic antioxidant defense systems are responsible for protecting the cell from ROS formation, but once the antioxidant system is disrupted and the redox status of cells is altered, ROS associated toxic effects and pathologies can be observed. However, ROS cause their pathologies through activation of signaling pathways and apoptosis, rather than direct oxidative damage (Schieber and Chandel, 2014). Yet increased levels of ROS within immune cells can result in hyperactivation of inflammatory responses, resulting in tissue damage and pathology (Mittal *et al.*, 2014). In a study , Raghubeer and colleagues (2017), described inflammation and apoptosis in human embryonic kidney cells (HEK293) exposed to OTA. In low concentrations of OTA, an anti-apoptotic and pro-inflammatory environment was found, but as OTA concentrations were increased, this environment changed to anti-inflammatory and pro-apoptotic (Raghubeer *et al.*, 2017).

In addition, it has been demonstrated that ROS generation increased by OTA promotes the activation of the apoptosis signaling pathway through the mitochondrial lipid peroxidation, promoting loss of mitochondria membrane potential, increasing membrane permeability (Bhat *et al.*, 2016). The lesional mechanisms promote changes in the Bcl-2 family, inducing the expression of Bax, facilitating the release of cytochrome C and the activation of caspase 3 in the cytosol (da Silva *et al.*, 2018). Based on these evidences, the toxic effects caused by OTA are the consequence of the disruption of the normal redox status and consequent reactive species production. Our results showed that the treatment with OTA significantly enhanced oxidative phosphorylation (OXPHOS) CI-Linked states, indicating a cellular attempt to compensate for the depletion of ATP caused by inhibition of the electron transporting chain, which was significantly inhibited by OTA treatment. Our results are in accordance with earlier studies, which demonstrated OTA exerts its effect on the mitochondrial respiration and oxidative phosphorylation through the impairment of the

mitochondrial membrane and inhibition of the succinate-supported electron transfer activities of the respiratory chain (Li *et al.*, 2019).

Moreover, in our study we investigated the effects of OTA on SOD and CAT activity, which are key enzymes of the mitochondrial endogenous antioxidant system. Our results showed that OTA significantly increased total SOD activity in low concentrations, which could indicate an effort to maintain the balance in cellular redox status, preventing oxidative damage. Despite being an antioxidant enzyme, an increase of SOD activity may occasionally induce oxidative stress due to the accumulation of peroxide. This occurs when the increase of SOD activity is not accompanied by increased activity of hydrogen peroxide-removing enzymes and is characterized by an increased SOD/CAT ratio (Pinho *et al.*, 2006). Our results showed an inhibition of CAT activity in low concentrations of OTA, above 1 µM, indicating an imbalance between SOD/CAT ratio, which could cause accumulation of hydrogen peroxide in the mitochondria and thus leading to oxidative stress. In the intermediary concentrations of OTA, we did not observed a significant change in total SOD activity, and in concentrations above 10 µM SOD activity was significantly reduced. SOD is the enzyme responsible for reducing the superoxide radical into hydrogen peroxide ($H_2O_2$), which is the substrate to CAT. When the cell has increased levels of SOD without a proportional increase in peroxidases, it faces a peroxide overload challenge. Peroxide can react with transitional metals and generate the radical hydroxyl, which is the most harmful radical (Pinho *et al.*, 2006). Moreover, in these concentrations oxidative damage to lipids was already observed by TBARS assay. These results are in accordance with Boesch-Saadatmandi (2008) study, which showed that OTA exposure decreases SOD activity in concentrations above 10 µM in porcine kidney tubular cells (LLC-PK1). Additionally, these authors also found an decrease in CAT, glutathione-S-transferase and glutathione-peroxidase in LLC-PK1 cells exposed to concentrations of OTA above 10 µM (Boesch-Saadatmandi *et al.*, 2008).

When compared the results PK-15 cells and porcine PBMCs, we observed that the toxic effects of both cell types were very similar in these *in vitro* experiments. The kidney is the main target organ for OTA toxicity, due to the toxicokinetics characteristics of this mycotoxin. The reabsorption of filtered and secreted OTA in kidneys retards its excretion and may lead to the accumulation of the toxin in the renal tissue, contributing to its renal toxicity (Pfohl-Leszkowicz and Manderville, 2007). These toxicokinetic features may explain why the kidney represents the main target of OTA even though other cell types, such as PBMCs, have similar susceptibility to this mycotoxin. Although OTA effects are well documented in human PBMCs (Periasamy *et al.*, 2016), as far as we know, this is the first study describing OTA cytotoxic effect in porcine PBMCs. In a review of the oxidative hypothesis regarding to OTA toxic effects, Tao and colleagues (2018), have shown that studies using different animal species must be performed to enlighten the mechanisms underlying the OTA related toxicity and understanding the relationship between the metabolism of OTA and oxidative stress (Tao *et al.*, 2018).

A better understanding of the toxicity and metabolic pathway of OTA *in vivo* and *in vitro* is necessary to establish strategies to prevent and combat the toxic effects caused by OTA in humans and animals. Our results showed that OTA causes loss in viability, increase in oxidative stress and lipid peroxidation in porcine PK-15 cells and fresh PBMCs, besides an imbalance in SOD activity and a decrease in CAT activity. Moreover, mitochondrial dysfunction was observed in PK-15 cells exposed to OTA and similar effects are caused in porcine kidney cells and PBMCs in consequence of OTA exposure regarding to cell viability and oxidative stress.

## Conflict of Interest Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Acknowledgements

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# 4 PROTOCOLO EXPERIMENTAL II

## CYTOPROTECTIVE EFFECTS OF CURCUMIN AND SILYMARIN ON PK-15 CELLS EXPOSED TO OCHRATOXIN A, FUMONISIN $B_1$ AND DEOXYNIVALENOL

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# Cytoprotective effects of curcumin and silymarin on PK-15 cells exposed to ochratoxin A, fumonisin $B_1$ and deoxynivalenol

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**Abstract**

Mycotoxins are toxic secondary metabolites produced by fungus which cause worldwide concern regarding food and feed safety. Ochratoxin A (OTA), fumonisin $B_1$ ($FB_1$) and deoxynivalenol (DON) are some of the main mycotoxins and oxidative stress is the main mechanism of toxicity. Thereby, this study investigates the *in vitro* cytoprotective effects of curcumin (CUR) and silymarin (SIL) - known for their strong antioxidant activity - in PK-15 cells exposed to OTA, $FB_1$ and DON. Pretreatment with CUR and SIL enhanced the viability of cells exposed to the mycotoxins ($P < 0.001$) and attenuated reactive oxygen species (ROS) formation by DON ($P < 0.01$), partially reduced ROS formation by $FB_1$ ($P < 0.001$), but not OTA. CUR significantly decreased apoptosis in cells exposed to DON ($P < 0.01$) but was not able to prevent apoptosis in cells exposed to OTA and $FB_1$. Whereas SIL was able to prevent apoptosis in PK-15 cells exposed to $FB_1$ and DON ($P < 0.01$) but was not able to decrease apoptosis in cells exposed to OTA. In summary, these data indicate that curcumin and silymarin are able to provide cytoprotection against toxicity induced by OTA, $FB_1$ and DON in PK-15 cells.

**Keywords:** Mycotoxins; Ochratoxin A; Fumonisin $B_1$; Deoxynivalenol; Curcumin; Silymarin.

## 1 Introduction

Mycotoxins are second metabolites produced by filamentous fungi that are toxic even in low concentrations to vertebrates and other groups of animals. Moreover, mycotoxins are the most commonly occurring natural food contaminant in human and animal feed. (BENNETT; KLICH, 2003; MARROQUIN-CARDONA et al., 2014). The animal production industry is most commonly affected by mycotoxins. Overall, most mycotoxins cause immunosuppression which leaves animals more vulnerable to diseases by weakening their immune system or making them less responsive to vaccinations. In acute cases, losses are related to mortality. Other subclinical effects may cause loss in productivity, reduce weight gains and interfere in feed efficiency (MARROQUIN-CARDONA et al., 2014).

Ochratoxin A (OTA), fumonisin $B_1$ ($FB_1$) and deoxynivalenol (DON) are some of the most common mycotoxins, produced by different fungus of genera *Aspergillus*, *Penicillium* and *Fusarium* (MARIN et al., 2013). OTA can be found in contaminated food grains and cereals, fruit, fruit products and coffee. The main mechanism of OTA toxicity consists of high production of reactive oxygen species (ROS), which leads to mitochondrial dysfunction, cellular damage and genotoxicity. The kidney is the organ most affected by OTA toxic effects due to its role to the mycotoxin excretion through the urine, allowing the toxin to exert its toxic effects on kidney tubules, impairing kidney function (KOSZEGI; POOR, 2016; RAGHUBEER et al., 2017). Fumonisins are mostly found in maize which are contaminated with *Fusarium verticillioides* and *Fusarium proliferatum*. $FB_1$ is the most important fumonisin from a toxicological perspective and its toxicity mechanism is based on the fact that $FB_1$ has a structure similar to sphingoid bases such as sphingosine, and can function as an inhibitor of ceramide synthase, therefore, this mycotoxin strongly inhibits the enzyme ceramide synthase that catalyzes the acylation of sphinganine and recycling of sphingosine.

The inhibition of CER synthase increases intracellular sphinganine and other sphingoid bases, which are highly cytotoxic compounds (LIU et al., 2019). Other studies have demonstrated the potential of $FB_1$ to promote oxidative stress with consequent increase in intracellular ROS levels and their cytotoxic effects, and induction of apoptosis (DOMIJAN et al., 2015; ABBES et al., 2016), $FB_1$ is also able to modulate the expression of enzymes of the antioxidant system and to increase MDA levels (DOMIJAN, ZELJEZIC, et al., 2007; ABBES et al., 2016; DA SILVA et al., 2018). DON is a type B trichothecene predominantly produced by *Fusarium graminearum* and *Fusarium culmorum* (BENNETT; KLICH, 2003). Ingestion of DON contaminated foods induces vomiting, anorexia, disturbance of cell signaling and differentiation, immunotoxicity and disturbance of gastrointestinal homeostasis (PESTKA, 2010; ABDEL-WAHHAB et al., 2015). Oxidative stress is considered the most important factor in the toxicity of DON. Previous studies demonstrate that DON induces damage to the cell membrane, chromosomes and DNA, increases lipid peroxidation and ROS in human peripheral blood mononuclear cells. The oxidative stress signaling pathway induced by DON has been suggested to be one of the mechanisms behind DNA fragmentation, cell death and apoptosis (FRANKIC et al., 2006; DA SILVA et al., 2018).

Several studies have demonstrated the ability of polyphenols to prevent and even neutralize the damage caused by various toxic agents, including mycotoxins, through their antioxidant activity (QIN et al., 2015; RAGHUBEER et al., 2015; ZHANG et al., 2016). Therefore, studies that confirm the effectiveness of these compounds in the treatment of mycotoxicosis which cause most damage to pig farming, are very relevant due to their potential application as a dietary supplement to these animals.

Curcumin is a yellow pigment extracted from the rhizome of the plant *Curcuma longa*, which has been used as a food additive and traditional medicine in Asia for a long time, and recently started to be consumed worldwide. Several studies have shown that curcumin is a

powerful inhibitor of oxidative stress, acting as a direct free radicals scavenger and removing superoxide and peroxide (ARAUJO; LEON, 2001; SHARMA et al., 2005; MOGHADAMTOUSI et al., 2014; VALLIANOU et al., 2015). Silymarin is a phenolic compound extracted from the fruits and seeds of the plant *Silybum marianum*. According to previous literature, silymarin has several activities such as hepatoprotective and regenerating, antioxidant, antifibrotic and inti-inflammatory. The hepatoprotective activity of silymarin is mainly due to the capture of free radicals produced by various events in cells, avoiding the damage caused by oxidative stress. (WELLINGTON; JARVIS, 2001; SCHUMANN et al., 2003; CROCENZI; ROMA, 2006; LIN et al., 2008; SANGEETHA et al., 2010).

In the past years, several studies have suggested that oxidative stress is the main mechanism of toxicity induced by mycotoxins. Therefore, this study investigates the in vitro cytoprotective effects of curcumin and silymarin, two phenolic compounds that are known for their strong antioxidant activity, on epithelial cells of porcine kidney from the PK-15 cell line. We believe that our results can provide some valuable insights into OTA, $FB_1$ and DON toxicity in porcine kidney cells. Moreover, the results also elucidate whether these compounds could be used as potential feed additives to prevent mycotoxins toxicity.

## 2 Materials and methods

### 2.1 Reagents and chemicals

The mycotoxins OTA, $FB_1$ and DON, the phenolic compounds curcumin and silymarin, Eagle's Minimum Essential Medium, antibiotics penicillin/streptomycin, trypsin/EDTA, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 2,7-Dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis,

MO, USA). Fetal Bovine Serum (FBS) was purchased from Vitrocell (Campinas, SP, Brazil) and Annexin V-FITC kit was purchased from BD Biosciences (USA). All other chemicals and reagents used in these experiments were of the highest purity.

## 2.2 Cell line and culture conditions

Cells of PK-15 cell line (ATCC, Rockville, MD, USA) were grown in 75-$cm^2$ culture flasks in a culture medium containing Eagle's Minimum Essential Medium with 5% heat-inactivated fetal bovine serum, 100 UI/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% $CO_2$ at 37 ºC. When cells reached confluence of ~80%-90%, the cells were trypsinized and plated in flat-bottom cell culture plates for performing cytotoxicity assays. All tests were performed in triplicates and the volume of mycotoxins and CUR and SIL stock solutions added to the treatments never exceeded 0.1% of the culture medium to avoid toxicity of DMSO. The PK-15 cell line selected for this study is based on previous studies which consider the kidney as a primary or secondary target in order to perform *in vitro* toxicological assessment of mycotoxins (LEI et al., 2013; CHEN et al., 2020).

## 2.3 Experimental design

To evaluate the toxicity of the tested mycotoxins as well as curcumin and silymarin, the MTT cell viability assay was performed using an initial range chosen based on previous literature. Moreover, the concentrations of mycotoxins that caused ~50% of decrease on PK-15 cell viability were chosen for further experiments. Following the same pattern, after performing MTT assay on PK-15 cells treated with different concentrations of curcumin and

silymarin, concentrations that did not show toxicity were chosen to evaluate the cytoprotective activity of these compounds on PK-15 cells exposed to OTA, $FB_1$ and DON. PK-15 cells were seeded into 96-well plates ($1 \times 10^4$ cells/mL) and incubated under conditions described above for 24 h to allow the cells to attach to the plates. After incubation, the medium was replaced with fresh medium containing the mycotoxins OTA (0.5 – 10 µM) (RAGHUBEER et al., 2015), $FB_1$ (10 – 100 µM) (SUN et al., 2015) or DON (0.01 – 2.5 µM) (ALASSANE-KPEMBI et al., 2013), and also CUR or SIL (1 – 10 µM), along with an untreated control group (CTR). The plates were incubated for 48 h and MTT assay was performed.

After the preliminary cytotoxicity evaluation, one concentration of each mycotoxin was chosen, along with three concentrations of CUR and SIL, to assess the cytoprotective capacity of these compounds. Cells were seeded and incubated under conditions described above and after 24 h of incubation, cells were pretreated with CUR (0.5, 1 or 2 µM) or SIL (1, 2.5 or 5 µM) and incubated for 6 h. After the incubation time, pretreated cells were exposed to the mycotoxins OTA (5 µM), $FB_1$ (50 µM) and DON (0.25 µM), along with a negative control (untreated cells) and a positive control (cells treated only with the mycotoxins). Plates were incubated for 48 h and the MTT assay was performed as described below. Based on the results obtained in this assay, the concentration of CUR and SIL that showed the greater cytoprotective effect against each mycotoxin tested was chosen for subsequent tests.

## 2.4 Analysis of cell viability using the MTT assay

The viability of PK-15 cells was evaluated based on the method described by Mosmann (1983) with slight modifications (MOSMANN, 1983). The cells were plated, treated and incubated for 48 h as previously described. Two hours before ceasing incubation,

20 mL of a 5 mg/mL MTT solution was added to each well and the incubation was continued. After the incubation time had finished, the plates were centrifuged at 400 g for 5 min at room temperature. The supernatant was carefully discarded and 200 mL of dimethyl sulfoxide (DMSO) was added into each well. The absorbance was measured at 590 nm in a microplate reader UV/Visible spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The viability of CTR group was calculated from the mean absorbance reading and represented as 100%.

2.5 Determination of ROS production by DCFH-DA staining

Reactive oxygen species (ROS) production was evaluated according to the method described by Amer and colleagues with slight modifications (AMER et al., 2003). PK-15 cells were seeded into 24-well plates, pretreated with CUR and SIL for 6 h and then exposed to the mycotoxins and incubated for 48 h as previously described. After the incubation time had finished, cells were trypsinized, harvested in microtubes and incubated with DCFH-DA (5 µM) probe for 30 min in the dark. The cells were washed with PBS and immediately subjected to flow cytometry (BD Accouri). Fifty thousand events were analyzed for each sample and green fluorescence intensity was collected in the FL-1 channel. The results were expressed as the relative mean of fluorescence emitted by cells stained with DCFH-DA.

2.6 Analysis of apoptosis by annexin V staining

PK-15 cells were seeded into 24-well plates, treated and incubated for 48 h as previously described. After the incubation period, cells were detached by trypsinization and collected in 0.5 mL of DMEM with 10% Fetal Bovine Serum. Afterwards, cells were centrifuged at 400 g during 5 min at 4 °C, resuspended and washed with ice-cold PBS,

centrifuged, and resuspended in ice-cold 1 × binding buffer (BB) to $5 \times 10^5$ cells/mL. Cells were incubated with 20 µL/mL Annexin V-FICT and 50 µg/mL propidium iodide (PI) solution according to the manufacturer instructions. As a result, four different groups of cells were obtained based on their stainability: those unstainable with annexin V or PI [annexin(-)/PI(-)]: viable cells (quadrant E3); those stainable with annexin V but unstainable with PI [annexin(+)/PI(-)]: early apoptotic cells (quadrant E4); those stainable with both annexin V and PI [annexin(+)/PI(+)]: late apoptotic cells (quadrant E2); and those unstainable with annexin V but stainable with PI [annexin(-)/ PI(+)]: primary necrotic cells (quadrant E1). The untreated population was used to define the basal level of apoptotic and dead cells. Following the acquisition of sample data (channel FL1 and FL3) on a BD Accuri™ C6 flow cytometer (BD Biosciences®), the sample results were generated in graphic and tabular format using FCAP array v 3.0.1 software. Appropriate fluorescence compensation was manually set for FL1 (annexin) and FL3 channel (PI) to avoid signal overlap. Fifty thousand events were acquired for each sample.

## 2.7 Statistical analysis

The results were evaluated statistically by analysis of variance (ANOVA) with Dunnet's test for the preliminary cytotoxicity tests and Tukey's test for all other in vitro analysis using the Graph Pad Prism version 6.0 software and were expressed as the mean ± standard error. The level of statistical significance was defined as $P < 0.05$. All experiments were performed in triplicates and in three different days.

## 3 Results

### 3.1 Preliminary toxicity of mycotoxins, CUR and SIL

The MTT assay was used to determine the concentrations of OTA, $FB_1$ and DON that caused ~50% of inhibition on PK-15 cell viability after 48 h exposure. As shown in Fig. 1, these concentrations were 5 µM for OTA (41,5% of inhibition), 50 µM for $FB_1$ (47.3% of inhibition) and 0.25 µM for DON (43.8% of inhibition). MTT assay was also used to determine the limit of non-cytotoxic concentration of CUR and SIL, which demonstrated that concentrations of CUR up to 5 µM as well as 10 µM of SIL did not present toxicity in PK-15 cell upon 48 h treatment (Fig. 1d and 1e).

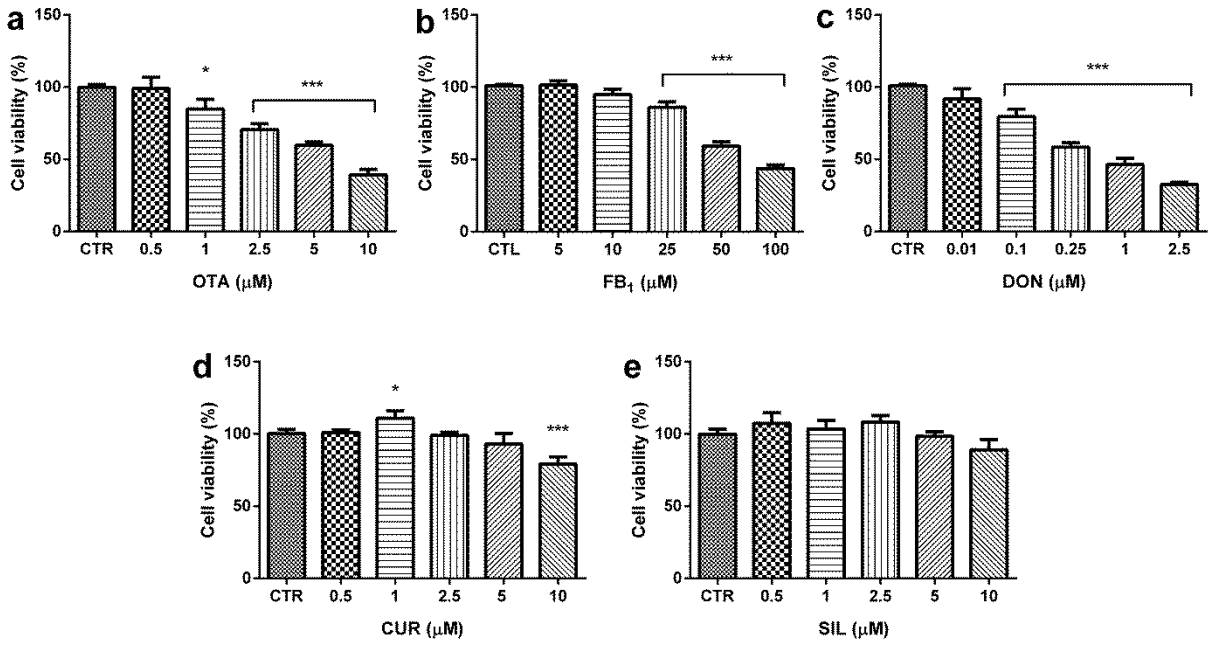


**Fig. 1** MTT cell viability assay of PK-15 cells exposed to different concentrations of the mycotoxins OTA (a), $FB_1$ (b) and DON (c), as well as the phenolic compounds curcumin (d) and silymarin (e) after 48 h exposure. Results are expressed as % of the control group and represent the mean ± SD. $*P < 0.05$, $***P < 0.001$ indicate statistical difference from CTR by One-way ANOVA followed by Dunnett test.

3.2 Protective effect of CUR and SIL on cell viability by MTT assay

The protective effect of CUR and SIL on PK-15 cells exposed to the mycotoxins is illustrated in Fig. 2. Pretreatment with both CUR and SIL in different concentrations was able to prevent cell viability loss caused by OTA, $FB_1$ and DON. Treatment with CUR significantly alleviates the inhibition of cell viability caused by exposure to 5 µM of OTA at the three concentrations tested (0.5, 1 and 2 µM), showing greater protection at the concentration of 1 µM (from 58.5% of cell viability in cells exposed only to OTA to 81.4% in cells pretreated with CUR for 6 h and then exposed to OTA for additional 48 h). Similarly, cells pretreated with SIL (1, 2.5 and 5 µM) and exposed to OTA also had an improvement in cell viability at all concentrations tested, with greater protection at 2.5 µM (from 58.5% in cells exposed only to OTA to 80.3% in pretreated cells). In PK-15 cells exposed to 50 µM of $FB_1$, pretreatment with CUR was able to improve cell viability at 1 and 2 µM, but not in the lowest concentration, showing greater protection at 1 µM (from 53.7% in cells only exposed to $FB_1$ to 77% in pretreated cells), while pretreatment with SIL was able to fully neutralize cell viability loss at the concentration of 2.5 µM (from 53.7% to 89.2% of cell viability), and also ameliorated cell viability at 5 µM (from 53.7% to 75.8%) but not at 1 µM. Meanwhile, PK-15 cells exposed to 0.25 µM of DON had an improvement with all concentrations of CUR and SIL pretreatment. Cell viability loss was statistically fully neutralized with pretreatment with 1 and 2 µM of CUR and 2.5 and 5 µM of SIL, with grater cell viability at 1 µM of CUR (from 57.2% to 89.4%) and 5 µM of SIL (from 57.2% to 88%). Therefore, the concentration of 1 µM of CUR was chosen to perform subsequent tests, while the concentration of SIL was 2.5 µM when used to protect cells against OTA and $FB_1$ and 5 µM to protect cells against DON.

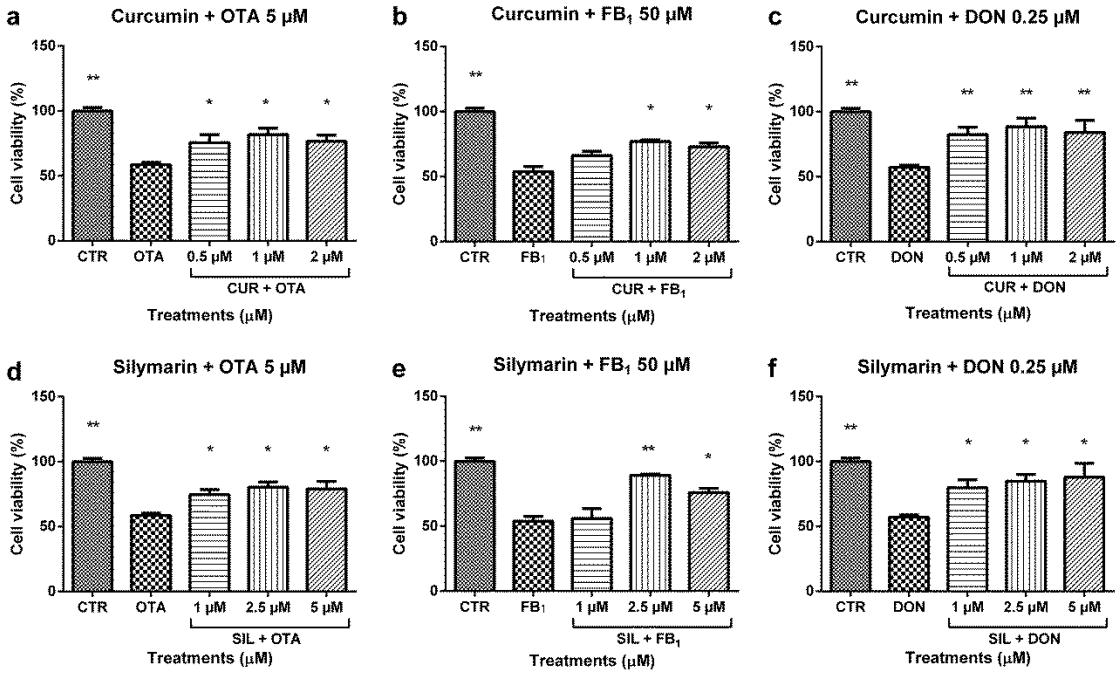


**Fig. 2** MTT cell viability assay of PK-15 cells pretreated with different concentrations of CUR (0.5, 1 or 2 µM) or SIL (1, 2.5 or 5 µM) for 6 h and exposed to the mycotoxins OTA (5 µM), $FB_1$ (50 µM) and DON (0.25 µM) for 48 h. **a** PK-15 cells pretreated with CUR exposed to OTA; **b** PK-15 cells pretreated with CUR exposed to $FB_1$; **c** PK-15 cells pretreated with CUR exposed to DON; **d** PK-15 cells pretreated with SIL exposed to OTA; **e** PK-15 cells pretreated with SIL exposed to $FB_1$; **f** PK-15 cells pretreated with SIL exposed to DON. Results are expressed as % of the control group and represent the mean ± SD. **P* < 0.01, ***P* < 0.001 indicate statistical difference from the group exposed only to the mycotoxins (OTA, $FB_1$ or DON) by One-Way ANOVA followed by Tukey test.

3.3 CUR and SIL effect on ROS production

The levels of intracellular ROS were quantified by flow cytometry by DCFH-DA assay. DCFH-DA probe can cross the cell membrane freely and in the presence of intracellular ROS it is oxidized to a fluorescent DCF. Compared to the control group, the fluorescence intensity was enhanced by the mycotoxins after 48 h of exposure, as shown on Fig. 3. OTA increased ROS production by 52.3%, however, pretreatment with neither CUR nor SIL were able to prevent the induction of ROS formation by OTA. PK-15 cells exposed to $FB_1$ had an increase of 97.4% intracellular ROS but although CUR and SIL significantly

reduced ROS (to 75.5% and 34.2% respectively), they did not completely attenuated ROS generation. On the other hand, PK-15 cells exposed to DON had an increase of 21.2% intracellular ROS compared to the control group, which was completely attenuated by pretreatment with CUR and SIL.

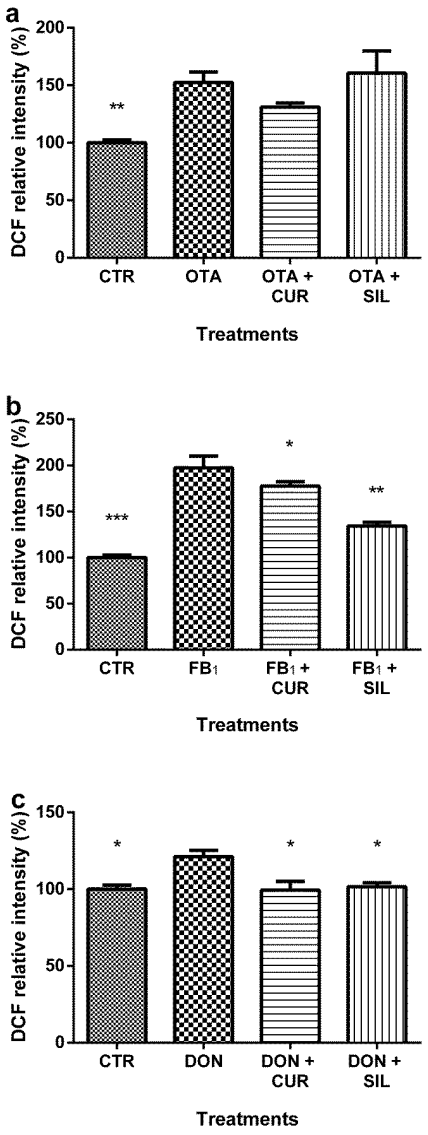


**Fig. 3** Reactive species generation of PK-15 cells after pretreatment with CUR or SIL and exposure to (a) OTA (5 µM), (b) $FB_1$ (50 µM) and (c) DON (0.25 µM) for 48 h. Results represent the mean ± SD of the fluorescence intensity of a representative experiment of 3 independent experiments carried out in triplicate. 50000 events were acquired and analyzed by flow cytometry. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$ indicate statistical difference from the group exposed only to the mycotoxins (OTA, $FB_1$ or DON) by One-Way ANOVA followed by Tukey test.

### 3.4 Impact of CUR and SIL pretreatment on mycotoxins-induced apoptosis

Flow cytometry based on the double staining reagents of PI and Annexin V-FITC (intact cells (FITC-/PI-), early apoptotic (FITC+/PI-) and late apoptotic/necrotic cells (FITC+/PI+)) demonstrated increased apoptotic rate (early apoptotic + apoptotic and necrotic cells) in PK-15 cells exposed to OTA (5 µM) from 2.9% in the CTR group to 5.6%, $FB_1$ (50 µM) increased apoptosis from 2.9% to 4.8%, while DON (0.25 µM) showed increase from 2.9% to 4.6%. Pretreatment with CUR (1 µM) significantly decreased apoptotic rates in cells exposed to DON from 4.6% to 3.5% but was not able to prevent apoptosis in PK-15 cells exposed to OTA and $FB_1$. Whereas pretreatment with SIL was able to prevent apoptosis in PK-15 cells exposed to $FB_1$ from 4.8% to 3% (2.5 µM of SIL) and from 4.6% to 3.6% in cells exposed to DON (5 µM of SIL), it was not able to decrease apoptosis in cells exposed to OTA, though.

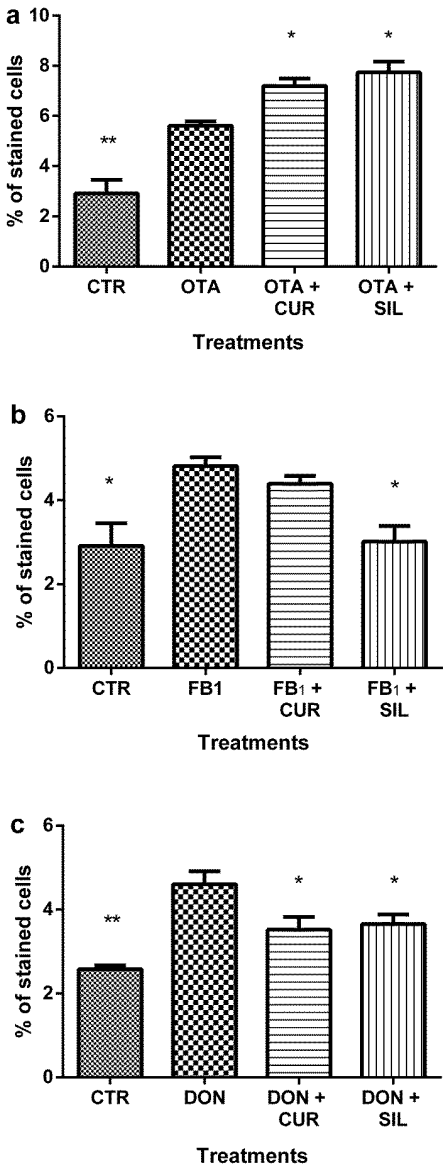


**Fig. 4** Percentage of Annexin V/PI positive PK-15 cells after pretreatment with CUR or SIL and exposure to (a) OTA (5 µM), (b) $FB_1$ (50 µM) and (c) DON (0.25 µM) for 48 h. Apoptotic cells were analyzed using the binding of Annexin V to phosphatidylserine on the surface of apoptotic cells and PI probe to differentiate the necrotic cells. Data are presented as mean ± SD from 3 independent experiments carried out in triplicate. 50000 events were acquired and analyzed by flow cytometry. $*P < 0.01$, $**P < 0.001$ indicate statistical difference from the group exposed only to the mycotoxins (OTA, $FB_1$ or DON) by One-Way ANOVA followed by Tukey test.

## 4 Discussion

The molecular mechanisms responsible to the toxic effects of the major mycotoxins are well known. In addition, oxidative stress along with generation of reactive species have been demonstrated to be implicated in mycotoxin toxicity (WANG et al., 2016; DA SILVA et al., 2018). Under normal conditions, ROS regulates intracellular signaling, however, under condition of excessive ROS production an imbalance between reactive species and the cell endogenous antioxidant system can be induced. This imbalance between reactive species and the antioxidant defense systems can cause damage to DNA, lipids and proteins, as observed upon exposure to mycotoxins (AHMED ADAM et al., 2017; DA SILVA et al., 2018). In this context, several studies have shown that phenolic compounds and other antioxidants have the ability to protect and even neutralize the damage caused by various toxic substances including mycotoxins (SORRENTI et al., 2013). Our study aimed to determine whether curcumin and silymarin could reduce or even prevent the damage induced by the mycotoxins OTA, $FB_1$ and DON in porcine kidney cells.

Our results demonstrated that pretreatment with both curcumin and silymarin prevented cell viability loss in porcine kidney cells exposed to OTA for 48 h, with an enhancement in cell viability up to 23.3%; however, none of the concentrations tested were able to fully neutralize OTA toxicity. On the other hand, pretreatment with neither CUR nor SIL could reduce ROS production or prevent cell apoptosis induced by OTA exposure upon 48 h of treatment. Similar results were found in a study investigating the protective effects of the phytoalexin resveratrol in human embryonic kidney cells (HEK293) exposed to OTA, where intracellular ROS was significantly enhanced after 48 h of treatment, however, other parameters as cellular viability loss and genotoxicity by single strand DNA breaks were significantly reduced (RAGHUBEER et al., 2015). The kidney is the main target of OTA

toxicity due to its role on this mycotoxin excretion by urine allowing the toxin to exert its toxic effects on kidney tubules and impairing kidney function (KOSZEGI; POOR, 2016), which could explain a major difficulty in protecting kidney cells from OTA toxicity.

When exposed to $FB_1$, PK-15 cells pretreated with CUR presented an enhance in cell viability and a significant inhibition in ROS production, while no significant reduction in the apoptotic rate was observed. On the other hand, SIL was able to fully prevent loss of cell viability and induction of apoptosis and significantly reduce ROS production. In this sense, apoptosis is a form of programmed cell death, which facilitates the removal of damaged cells, whereas $FB_1$ is known for promoting apoptosis by inhibiting ceramide synthase, leading to intracellular accumulation of sphingolipids which are pro-apoptotic, cytotoxic growth inhibitors and immunotoxic (MARASAS et al., 2004) and also by ROS generation (DOMIJAN et al., 2015; HASSAN et al., 2015; ABBES et al., 2016). Therefore, the antiapoptotic effect presented by SIL on PK-15 cells exposed to $FB_1$ could be due to its immunoprotection besides its antioxidant activity, since it was observed that SIL inhibited immunotoxic effects caused by mycotoxins (KHATOON et al., 2013). Nevertheless, it is still unclear if oxidative stress emerges as a consequence rather than as a causal mechanism of $FB_1$ toxicity (WANG et al., 2016; DA SILVA et al., 2018).

Pretreatment with both CUR and SIL in porcine kidney cells were able to completely protect cells against loss of cell viability and ROS production in cells exposed to DON for 48 h and were also able to reduce apoptotic rate. It is important to highlight that our results are in accordance with a study performed in rats, which showed that silymarin nanoparticles were able to protect the liver against hepatotoxicity induced by DON by modulating antioxidant enzymes and diminishing genotoxicity (ABDEL-WAHHAB et al., 2018). DON is known for inducing oxidative stress leading to DNA damage, inhibiting DNA, RNA and protein

synthesis, increasing lipid peroxidation and protein damage (AUDENAERT et al., 2013; STRASSER et al., 2013; MISHRA et al., 2014; YANG et al., 2014).

Curcumin and silymarin are phytochemicals known for their antioxidant activity. Curcumin has shown the capacity to counteract the oxidative stress caused by zearalenone in porcine granulosa cells in an in vitro study by modulating the gene expression and decreasing free radicals in cells exposed to mycotoxins (QIN et al., 2015). In addition, curcumin was also able to decrease aflatoxin $B_1$ ($AFB_1$) induced hepatotoxicity and hepatocarcinogenicity in rats by modulating antioxidant enzymes (EL-AGAMY, 2010; EL-BAHR, 2015; POAPOLATHEP et al., 2015). As to silymarin, in vivo studies demonstrated the ability of this phytoalexin to prevent liver damage caused by mycotoxins in poultry and rodents. In BALB/c mice receiving $FB_1$ contaminated feed, silymarin was able to reverse liver damage caused by this mycotoxin (HE et al., 2004; SOZMEN et al., 2014). In rats, silymarin was able to protect liver cells from damage caused by $AFB_1$ by improving the membrane transport, the electron transport chain, gene transcription and oxidative stress (RASTOGI et al., 2000). Similarly, in chicken, silymarin was effective in protecting against liver damage caused by $AFB_1$ (GRIZZLE et al., 2009) and along with vitamin E, against immunotoxic effects caused by OTA (KHATOON et al., 2013).

In conclusion, our findings suggest that curcumin and silymarin are both able to reduce the toxicity of the mycotoxins OTA, $FB_1$ and DON in the PK-15 cell line, but most effectively against $FB_1$ and DON toxicity. In fact, curcumin protected cells by reducing the cell viability loss induced by all mycotoxins tested, reducing the formation of ROS induced by $FB_1$ and even neutralizing ROS formation induced by DON and also reducing apoptosis caused by this mycotoxin. Moreover, pretreatment with silymarin inhibited the cell viability loss caused by OTA and fully neutralized the cell viability loss caused by $FB_1$ and DON, inhibited the formation of ROS induced by $FB_1$ and neutralized ROS formation caused by

DON and finally neutralized apoptosis caused by $FB_1$ and inhibited apoptosis induced by DON. Thus, our results indicate that these phytochemicals may be used to prevent nephrotoxicity caused by mycotoxins present in animal feed.

## Conflict of interest statement

There are no conflicts of interests to declare.

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## 5 PROTOCOLO EXPERIMENTAL III

CYTOPROTECTIVE EFFECTS OF CURCUMIN AND SILYMARIN IN MICE EXPOSED TO OCHRATOXIN A AND FUMONISIN $B_1$

Manuscrito formatado segundo as normas do periódico *Toxicological sciences*, na qual será submetido

[1]Certificado de aprovação pela CEUA no anexo D.

# Cytoprotective effects of curcumin and silymarin in mice exposed to ochratoxin A and fumonisin $B_1$

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**Abstract**

Mycotoxins are toxic secondary metabolites produced by fungus which have caused worldwide concern regarding food and feed safety. Ochratoxin A (OTA) and fumonisin $B_1$ ($FB_1$) are some of the most toxic mycotoxins and it has been demonstrated that oxidative stress is the main mechanism of toxicity induced by these mycotoxins. Thereby, this study investigated the *in vivo* hepatoprotective and nephroprotective potential of curcumin (CUR) and silymarin (SIL), two phenolic compounds with strong antioxidant activity, in adult male Swiss mice exposed to OTA and $FB_1$. Twelve groups of mice (n=5) were treated orally for 14 days as follows: the control groups; CUR (80 mg/kg b. w.), SIL (100 mg/kg b. w.) or CUR+SIL-treated groups; $FB_1$-treated group (1.5 mg/kg b. w.); OTA-treated group (1.0 mg/kg b. w.); CUR, SIL and CUR+SIL plus $FB_1$-treated groups; and CUR, SIL and CUR+SIL plus OTA-treated groups. Blood and tissue samples of liver and kidney were collected for different analyzes. The results revealed that OTA and $FB_1$ induced oxidative stress, with an increase in malondialdehyde levels and carbonyl content in liver and kidney, but these toxic effects were mitigated in animals pre-treated with CUR and SIL. Besides, CUR and SIL prevented the inhibition of catalase activity and improved liver and kidney function in mice exposed to OTA and $FB_1$. Moreover, histological tests showed a reduction in tubular necrosis in kidney and hepatocytes necrosis in liver due to the pre-treatment with CUR an SIL. In summary, these results demonstrate the potential of curcumin and silymarin to reduce the toxic effects induced by OTA and $FB_1$ in liver and kidney of mice exposed to these mycotoxins.

**Key-words:** Mycotoxins; Ochratoxin A; Fumonisin $B_1$; Curcumin; Silymarin

## Introduction

Mycotoxins are second metabolites produced mainly by the mycelial structure of filamentous fungi that are toxic to human beings and other animal groups (Bennett and Klich, 2003; Hussein and Brasel, 2001). All mycotoxins are low molecular weight natural products that exert their toxicity even in low concentrations (Hussein and Brasel, 2001). They represent the most commonly occurring natural contaminant in human and animal feed (Marroquin-Cardona et al., 2014). Ochratoxin A (OTA) and fumonisin $B_1$ ($FB_1$) are some of the most prevalent mycotoxins, produced by different fungus of genera *Aspergillus*, *Penicilium* and *Fusarium* (Marin et al., 2013). The molecular mechanisms responsible for the toxic effects of the major mycotoxins are well established and oxidative stress and generation of free radicals have been shown to be implicated in mycotoxin toxicity (da Silva et al., 2018; Wang et al., 2016).

OTA was first found in the Balkan region, however, it can be detected practically in all territories of the world. OTA is present at all stages of the food chain (cereals, meat, fruits, wine, beer, coffee) and it is accumulated in animal feed and in human food due to the favorable weather conditions and microclimate, and to improper storage of food components (Marroquin-Cardona et al., 2014; van der Merwe et al., 1965). Previous studies demonstrated that the presence OTA in food may be associated with the chronic tubulo-interstitial kidney disease called Balkan Endemic Nephropathy (BEN) (Pfohl-Leszkowicz and Manderville, 2007). The main mechanism of OTA toxicity is not completely understood, but the most documented hypothesis is an increased production of reactive oxygen species (ROS), which leads to mitochondrial dysfunction, cellular damage and genotoxicity (da Silva et al., 2018; Koszegi and Poor, 2016). The kidney is the organ most affected by OTA toxic effects due to

its role on this mycotoxin excretion by urine, allowing the toxin to exert its toxic effects on kidney tubules, impairing kidney function (Koszegi and Poor, 2016; Raghubeer et al., 2017).

Fumonisins are commonly found in maize contaminated with *Fusarium verticillioides* and *Fusarium proliferatum*. Among the fumonisins, $FB_1$ is the most important from a toxicological perspective (Norred et al., 1992). Multiple molecular mechanisms have been identified to be involved in $FB_1$-induced toxicities, which include accumulation of intracellular free sphinganine due to perturbation of sphingolipid metabolism, induction of oxidative stress, activation of endoplasmatic reticulum stress and MAPKs, modulation of autophagy, and alteration of DNA methylation (Abbes et al., 2016; Domijan et al., 2015; Liu et al., 2019), in addition, $FB_1$ is able to modulate the expression of antioxidant system enzymes and increase MDA levels (Abbes et al., 2016; da Silva et al., 2018; Domijan et al., 2007).

In the past years, several studies have suggested that antioxidant compounds may minimize end even neutralize oxidative stress and its consequent negative physiological effects, which are suggested as the main mechanisms of toxicity induced by mycotoxins *in vitro* (Ledur and Santurio, 2020; Qin et al., 2015; Raghubeer et al., 2017; Sang et al., 2016) and *in vivo* (Abdel-Wahhab et al., 2018; Khatoon et al., 2013). In this context, curcumin and silymarin are two phenolic compounds known for their strong antioxidant and regenerative activities.

Curcumin is a yellow pigment extracted from the rhizome of the plant *Curcuma longa*, which has been used as a food additive and traditional medicine in Asia for a long time, and nowadays it is being consumed worldwide. Curcumin is described as a powerful inhibitor of oxidative stress, acting as a direct free radicals scavenger and removing superoxide and peroxide (Araujo and Leon, 2001; Moghadamtousi et al., 2014; Sharma et al., 2005; Vallianou et al., 2015). Silymarin is extracted from fruits and seeds of the herbaceous plant

*Silybum marianum*, and has been described as hepatoprotective and regenerating, antioxidant, antifibrotic and inti-inflamatory. The hepatoprotective activity of silymarin is mainly due to the capture of free radicals produced by various events in cells, avoiding the damage caused by oxidative stress. (Crocenzi and Roma, 2006; Lin et al., 2008; Sangeetha et al., 2010; Schumann et al., 2003; Wellington and Jarvis, 2001).

As human and animal exposure to mycotoxins is unavoidable due to their prevalent presence in crops, effective ways to mitigate their harmful impacts are necessary. Therefore, this study investigated the protective effects of curcumin and silymarin against the toxic effects of OTA and $FB_1$ on liver and kidney of mice. We believe our results will provide valuable insight about the potential use of curcumin and silymarin as feed addictive to prevent the toxicity induced by OTA and $FB_1$ consumption.

## Materials and methods

### *Reagents and chemicals*

The mycotoxins OTA and $FB_1$, the phenolic compounds curcumin and silymarin, and 2,7-Dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in these experiments were of the highest purity.

### *Animals*

Adult male Swiss mice (25 - 30 days; 25−30 g) were obtained from the Central Animal House of the Federal University of Santa Maria. The animals were maintained at a

constant temperature (23 ± 1°C) on a 12 - hour light/dark cycle with free access to food and water. All animal procedures were carried out with the approval of the Institutional Animal Care and Use Committee of the Federal University of Santa Maria (protocol number: 1185101117). All efforts were made to minimize the number of animals used in this study as well as their suffering.

*Experimental protocol*

To assess the protective effects of curcumin and silymarin against the toxic effects of OTA and $FB_1$ on liver and kidney of mice, 60 animals were randomly distributed into 12 groups (5 mice/group). After the acclimatization period of one week, the animals were treated orally for 14 days, the treatment consisted of a single daily intragastric administration by gavage, as summarized in Table 1. The experimental protocol was divided into two stages. In the first 7 days, the animals received only CUR and/or SIL, and the positive controls of mycotoxins received only vehicle (canola oil). In the second stage (7 remaining days) the animals received the treatment of CUR and/or SIL, concomitantly with the administration of the mycotoxins, which were administered every 48 hours, on days 8, 10, 12 and 14 of the experimental protocol. The animals were observed daily for signs of intoxication such as changes in urination, diarrhea, inappetence and apathy, as well as signs of suffering and pain, like arched posture, difficulty breathing, increased heart or respiratory rate, tremors, spasms, bristly fur and weight loss. At the end of the treatment period (i.e. day 15), all animals were subjected to anesthesia and blood samples were collected by cardiac puncture. After the blood collection and euthanasia of the animals, liver and kidneys were collected for histopathological evaluation and determination of the oxidative stress profile.

**Table 1:** Experimental groups and their respective treatments.

| Groups | Treatments |
|---|---|
| Group 1 | Vehicle |
| Group 2 | CUR |
| Group 3 | SIL |
| Group 4 | CUR + SIL |
| Group 5 | $FB_1$ |
| Group 6 | OTA |
| Group 7 | CUR + $FB_1$ |
| Group 8 | CUR + OTA |
| Group 9 | SIL + $FB_1$ |
| Group 10 | SIL + OTA |
| Group 11 | CUR + SIL + $FB_1$ |
| Group 12 | CUR + SIL + OTA |

Vehicle: canola oil
CUR: curcumin (80 mg/kg b. w.) (Poapolathep et al., 2015).
SIL: silymarin (100 mg/kg b. w.) (Sozmen et al., 2014).
$FB_1$: fumonisin B1 (1.5 mg/kg b. w.) (Sozmen et al., 2014).
OTA: ochratoxin A (1.0 mg/kg b. w.) (Yang et al., 2019).

*Serum biochemical analysis*

After the blood collection, the serum samples were separated using cooling centrifugation and stored at -20 °C until analysis. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine and urea were determined by a semiautomatic analyzer (BA-88A; Mindray, China) using commercial kits (Labtest Diagnóstica S.A., Brazil).

*Protein determination*

Protein was measured by the Coomassie blue method according to Bradford (1976) using serum albumin as standard.

*Determination of lipid peroxidation*

The liver and kidney index of lipid peroxidation was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) as previously described by Ohkawa et al. (1979) with some modifications. In short, 200 µL of serum or standard (malondialdehyde [MDA] 0.03 mM) was added to the reaction mixture containing 1 mL of 1 % ortho-phosphoric acid and 0.25 mL of an alkaline solution of thiobarbituric acid-TBA (final volume 2.0 mL) followed by 45 min of heating at 95 °C. After cooling, the samples were read at 532 nm against the blank of the standard curve. The results are expressed as ηmol of MDA/g of protein.

*Quantification of the Protein Carbonyl Content*

Protein oxidation was measured by an estimation of the carbonyl groups in tissue homogenate performed according to the method of Levine et al. (1990), with slight modifications. This method is based on the quantification of the protein carbonyl by reaction with 2,4-dinitrophenylhydrazine (DNPH) in acidic medium. First, proteins were precipitated using 0.5 mL of 10 % trichloroacetic acid (TCA) from 1 mL of tissue homogenate and were centrifuged at 3500 rpm for 15 min, discarding the supernatant. 0.5 mL of 10 mmol/L DNPH in 2 mol/L HCl was added to precipitate the protein and was incubated at room temperature for 30 min. After incubation, 0.5 mL of 10 % TCA was added to the protein precipitate, and the tubes were centrifuged at 3500 rpm for 15 min. After discarding the supernatant, the precipitate was washed twice with 1 mL of ethanol/ethylacetate (1:1), centrifuging out the supernatant to remove the free DNPH. The precipitate was dissolved in 1.5 mL of a protein dissolving solution (2 g sodium dodecyl sulfate and 50 mg ethylenediamine tetraacetic acid in 100 mL 80 mmol/L phosphate buffer, pH 8.0) and incubated at 37 °C for 10 min. The color

intensity of the supernatant was measured using a spectrophotometer at 370 nm against 2 mol/L HCl. The carbonyl content was calculated by using the molar extinction coefficient (21 $\times 10^3$ L/mol cm), and the results were expressed as ηmol of carbonyl/mg of protein.

*Quantification of ROS*

The formation of reactive species from liver and kidney was estimated according to Ali et al. (1992). An aliquot of 50 µL of homogenate was incubated with 10 µL of 2',7′-dichlorofluorescein-diacetate (DCFH-DA; 7 µM) in the dark. Reactive oxygen species (ROS) levels were determined by the fluorescence method. The oxidation of DCFH-DA to dichlorofluorescein (DCF) was measured for intracellular ROS detection. The intensity of DCF fluorescence emission was recorded, and 488 and 525 nm of excitation 60 minutes after the addition of DCFH-DA in the medium and the oxidized DCF was determined using a standard curve, and the results were expressed as U DCF/mg protein.

*Catalase activity*

Catalase (CAT) enzyme activity in tissue homogenate was measured with the method of Aebi (1984) with slight modifications. For the determination of the CAT activity, a 20 µL aliquot of the samples was added to a cuvette, and the spectrophotometric determination was started by the addition of 70 µL of freshly prepared 0.3 mol/L $H_2O_2$ in potassium phosphate buffer (50 mM, pH 7.0) to give a final volume of 1 mL. The rate of the $H_2O_2$ reaction was monitored at 240 nm for 2 min at room temperature. The CAT activity was calculated using the molar extinction coefficient (0.0436 $cm^2$/µmol), and the results were expressed as nmol of CAT/mg of protein.

*Histopathological analysis*

Liver and kidney samples from all animals were collected to evaluate lesions and other abnormalities. For histopathologic analysis, the samples were fixed in neutral 10% formaline solution, embedded in paraffin and cut into sections. After deparaffinization, the sections were stained with hemotoxylin-eosin (H&E) method for microscopic examination.

*Statistical analysis*

The results were evaluated statistically by two-way analysis of variance (ANOVA) with Tukey's test using the Graph Pad Prism version 6.0 software and were expressed as the mean ± standard error. The level of statistical significance was defined as $P < 0.05$.

**Results**

*Serum biochemical analysis*

An increase in the activity of ALT, AST and ALP in the serum of animals exposed to OTA and $FB_1$ was observed. As shown in Fig.1, serological levels of ALT and AST were significantly reduced by the treatment with both CUR and SIL alone or combined, while the levels of ALP were reduced by both compounds in mice exposed to $FB_1$, and only by SIL or SIL and CUR combined in mice exposed to OTA. The creatinine levels in mice exposed to OTA and $FB_1$ were increased, but were completely neutralized by all treatments in animals exposed to $FB_1$ and by the treatment of CUR and SIL alone in animals exposed to OTA. CUR and SIL combined were able to partially neutralize the creatinine levels increase in mice

exposed to OTA. The urea determined in mice serum was increased only in animals exposed to OTA, but no significant alteration was observed in animals from all other groups.

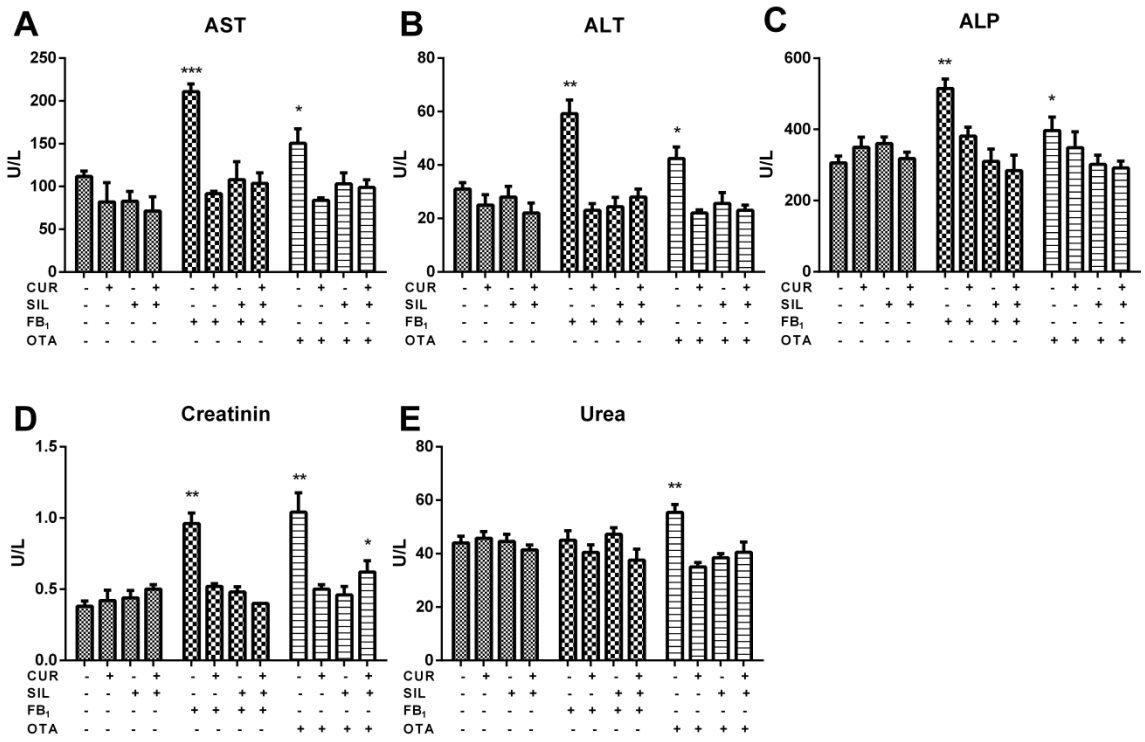


**Fig. 1:** Effect the pre-treatment of curcumin (CUR) and silymarin (SIL) on blood biochemical parameters of mice (n=5) exposed to OTA and $FB_1$. Serum levels of (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT),(C) alkaline phosphatase (ALP), (D) creatinine and (E) urea. Data are shown as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicate statistical difference from control by Two-way ANOVA followed by Tukey test.

*Oxidative stress parameters*

The results of liver and kidney oxidative stress parameters are shown in Figure 2. The TBARS assay demonstrated that lipid peroxidation was increased by both mycotoxins in the liver (Fig. 2A) and in the kidney (Fig. 2B) and the pre-treatment with curcumin and silymarin

alone reduced the MDA levels to same levels as observed in the control group, as the pre-treatment with CUR and SIL combined reduced the MDA levels in a lesser degree.

The determination of the carbonyl groups revealed the protein oxidation in liver and kidney (Fig. 2C and 2D, respectively). Our results demonstrated a slight increase in protein oxidation in the liver by the exposure to OTA and $FB_1$ in the liver, which was attenuated by the treatment with CUR and SIL. In the kidney, the protein oxidation caused by the mycotoxins was more increased, but was equally neutralized by the treatment with CUR and SIL.

The formation of reactive species on liver and kidney in mice exposed to OTA and $FB_1$ was considerably increased, especially in the kidney. CUR and SIL were both able to neutralize the ROS formation in the liver (Fig. 2E), but did not fully neutralized ROS formation in the kidney (Fig. 2F) of mice exposed to the mycotoxins. In this organ, CUR neutralized ROS formation caused by both OTA and $FB_1$, but SIL did not show the same effect nor alone nor in combination with CUR in animals exposed to OTA and showed a slight decrease in ROS in animals exposed to $FB_1$ when administered alone and a more considerable effect when administered in combination with CUR.

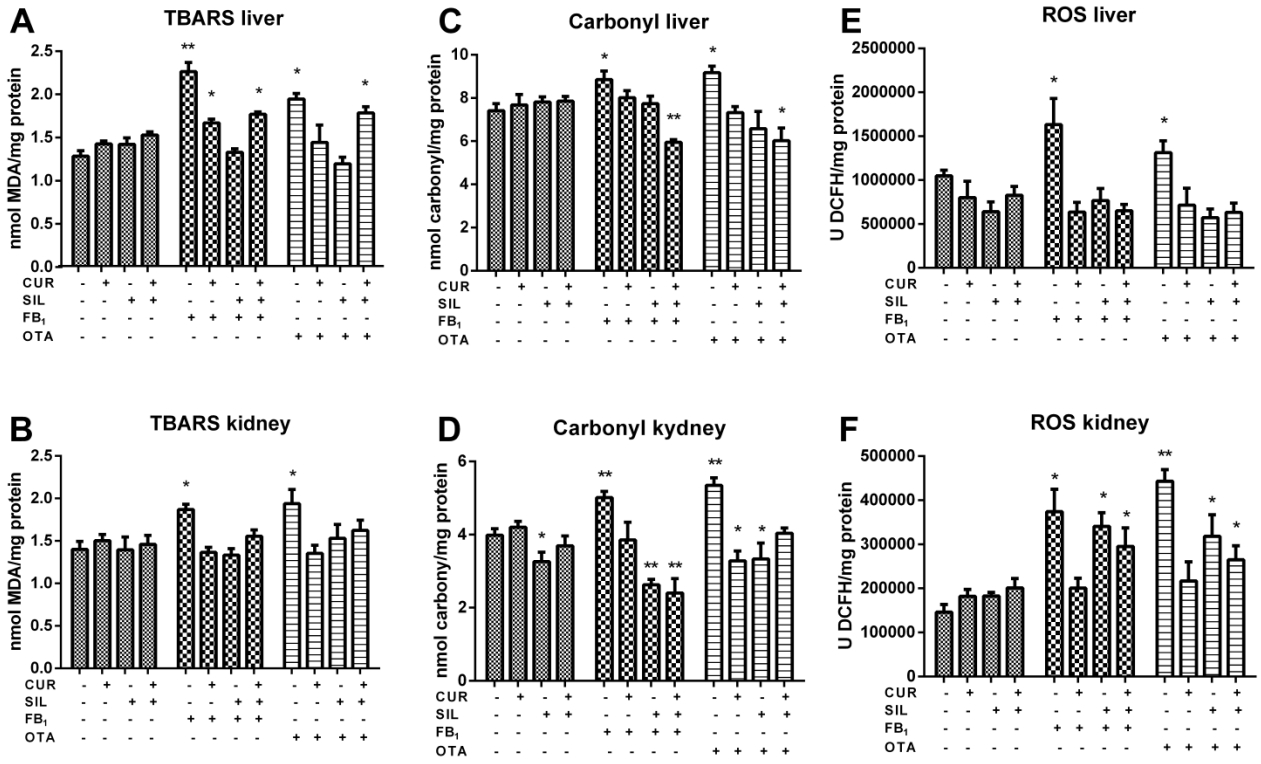


**Fig. 2:** Effect of CUR and SIL on oxidative stress parameters in liver and kidney of mice (n=5) exposed to OTA and $FB_1$. The MDA levels were determined by TBARS assay in liver (A) end kidney (B) tissue; carbonyl content was determined in the liver (C) and kidney (D) tissue; and reactive oxygen species (ROS) from liver (E) and kidney (F) tissue were determined. Data are shown as the mean ± SD. *P < 0.05, **P < 0.01, indicate statistical difference from control by Two-way ANOVA followed by Tukey test.

*CAT activity*

The activity of the systemic antioxidant enzyme catalase (CAT) in liver and kidney was significantly inhibited in the groups exposed to the mycotoxins, as seen in Fig. 3. In the liver SIL was able to neutralize the enzymatic activity reduction alone and in combination with CUR, but CUR alone was not able to mitigate the effects of the mycotoxins. In kidney, we observed a similar pattern, but just SIL and CUR in combination were able to neutralize the loss of catalase activity caused by $FB_1$, while SIL and SIL + CUR were able to mitigate the enzymatic activity inhibition caused by OTA.

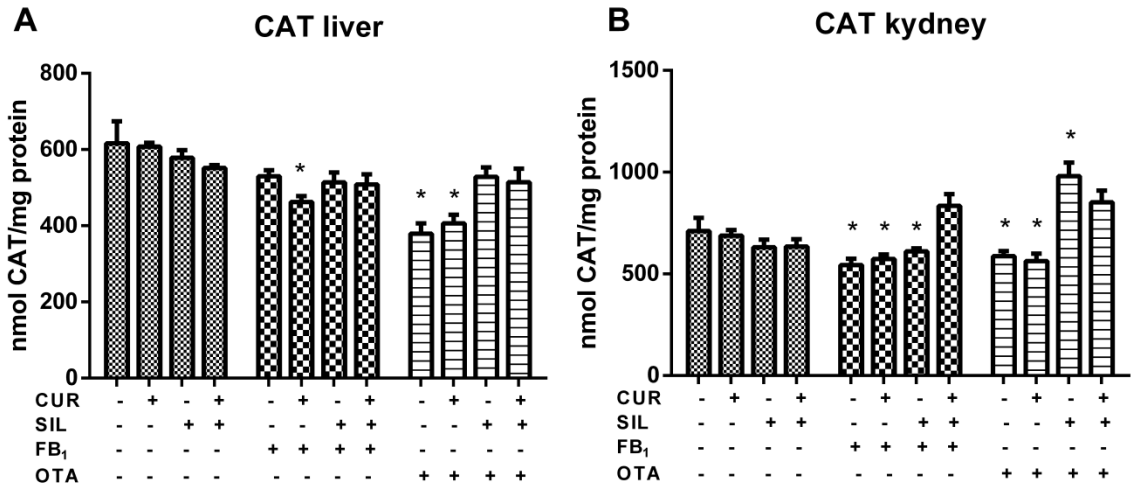


**Fig. 3:** Effect of CUR and SIL on CAT activity in (A) liver and (B) kidney of mice (n=5) exposed to OTA and $FB_1$. Data are shown as the mean ± SD. *P < 0.05, indicate statistical difference from control by Two-way ANOVA followed by Tukey test.

*Histological findings*

Histological examination revealed that liver and kidneys from the control group and CUR and SIL control groups were normal in appearance with typical histological structures. In the kidney of animals exposed to OTA and $FB_1$ we observed mild and moderate tubular necrosis, besides, moderate congestion was observed in kidneys of the $FB_1$ group. Also, necrotic cells were observed in the liver of animals from OTA and $FB_1$ groups. In the other hand, in the liver of animals from the groups treated with CUR and SIL and with the combination of these compounds and exposed to OTA and $FB_1$ we did not observe the necrotic hepatocytes as observed in the groups exposed to mycotoxins alone. However, CUR and SIL combined did not prevent the histological damage in the kidneys of mice exposed to the mycotoxins.

**Discussion**

In the current study, we investigated the protective effects of curcumin and silymarin singly or in combination in the kidney and in the liver of mice exposed to the mycotoxins OTA and $FB_1$. A common toxicological mechanism underlying the physiological consequences of the exposure to both mycotoxins, either as the cause or as a consequence of the toxic effects, is oxidative stress (Heussner and Bingle, 2015; Norred et al., 1992). As a matter of fact, the imbalance between free radicals and the antioxidant defense systems caused by mycotoxins can result in damage to DNA, proteins and lipids (da Silva et al., 2018). As the human and animal exposure to mycotoxins seems unavoidable (Heussner and Bingle, 2015), in the recent years several studies concerned in mitigating the physiological toxicity of mycotoxins using different compounds have been reported, among which the antioxidants have been highlighted.

Several studies reported oxidative damages in the liver due to exposure to mycotoxins (Bennett and Klich, 2003; Liu et al., 2019; Poapolathep et al., 2015) and both OTA and $FB_1$ caused an increase in the oxidative stress parameters tested in this study in the liver of mice exposed to these mycotoxins. Our data indicated an increase in MDA formation and its consequent lipid peroxidation, an increase in the content of carbonyl products, indicating damage to protein and also an increase in ROS formation. However, both CUR and SIL were able to mitigate these effects. In general, CUR and SIL played a similar role in protecting the liver of against the mycotoxins toxicity, but the lower MDA levels were achieved in the liver of mice treated with SIL alone, which completely mitigated the lipid peroxidation, indicating that SIL could offer a better protection against mycotoxin-induced liver injury. A previous study demonstrated that pretreatments with SIL prevented MDA formation and protected cells form oxidative stress by up-regulating enzymes from the antioxidant system in hepatocytes

exposed to OTA (Yu et al., 2018). On the other hand, CUR has already been reported in several studies as an hepatoprotective compound due to its antioxidant activity against the toxicity induced by $AFB_1$ in different animal models and *in vitro* studies (Nayak and Sashidhar, 2010; Poapolathep et al., 2015; Wang et al., 2018), however, the protective effect of CUR has not been well elucidated with other mycotoxins in animal models.

The kidney is the main target organ for OTA and nephrotoxicity has also been related as consequence to intoxication by $FB_1$ (da Silva et al., 2018; Koszegi and Poor, 2016). In the present study, an increase in all oxidative parameters tested was observed in the kidney of mice exposed to OTA and $FB_1$, indicating a significant nephrotoxicity of these mycotoxins, with increased MDA formation, an increased content of carbonyl products and also an increase in ROS formation. Although SIL was not able to reduce the ROS levels in the kidney of animals exposed to $FB_1$, it was able to partially reduce ROS levels in mice exposed to OTA, whereas CUR completely attenuated ROS formation in animals exposed to both mycotoxins. In the other hand CUR and SIL were able to mitigate MDA and carbonyl content. The protective effect of CUR and SIL in kidney cells exposed to $FB_1$ and OTA has been demonstrated *in vitro* in a previous study (Ledur and Santurio, 2020), and recently the protective effect of CUR in OTA-induced kidney damage has been reported and the antioxidant activity of CUR was pointed as responsible for these effects (Damiano et al., 2020).

Catalase (CAT) is a mitochondrial enzyme of the endogenous antioxidant system which plays a key role in the detoxification of free radicals, being the responsible for reducing hydrogen peroxide. If there is an inhibition of CAT, cells face a peroxide overload challenge. Peroxide can react with transitional metals and generate the radical hydroxyl, which is the most harmful radical (Pinho et al., 2006). An inhibition of CAT was noted in the liver of mice exposed to OTA and in the kidney of mice exposed to OTA and $FB_1$. This inhibition of CAT

in animals exposed to OTA was neutralized by the treatment with SIL and also by CUR and SIL combined. Whereas in the kidney of animals exposed to $FB_1$ only the treatment with the combination of CUR and SIL was able to increase CAT activity to the control levels. Our results are in accordance with a study performed in rats, which showed that SIL nanoparticles were able to protect the liver against hepatotoxicity induced by the mycotoxin deoxynivalenol (DON) by modulating antioxidant enzymes and diminishing genotoxicity (Abdel-Wahhab et al., 2018).

The histological findings observed in the present study revealed moderate tubular necrosis and moderate congestion in the kidneys of mice exposed to OTA and $FB_1$. Also, necrotic cells were observed in the liver of animals exposed to OTA and $FB_1$. CUR and SIL, alone and in combination, avoided the appearance of necrotic cells in the liver, whereas CUR and SIL combined did not prevent the histological damage in the kidneys of mice exposed to the mycotoxins. Similar findings were observed by Damiano et al. (2020) in a study that reported that the treatment with CUR showed a reduction in the number of glomerular and tubular necrosis. It has been reported that SIL significantly diminished the number of apoptotic hepatocytes in the liver of mice exposed to $FB_1$ and cellular regeneration was observed while decreasing cellular damage (He et al., 2004; Sozmen et al., 2014). Besides the antioxidant activity observed in this study, this protective effect of SIL can also be explained by the stimulatory effect of SIL in the regeneration of damaged liver tissues (He et al., 2004).

Consistent with other studies (Damiano et al., 2020; He et al., 2004; Yu et al., 2018), we observed an increase in the activity of ALT, AST and ALP in the serum of animals exposed to OTA and $FB_1$, with greater levels observed with the exposure to $FB_1$ that to OTA, indicating that hepatic damage was induced by the mycotoxins. However, the levels of ALT and AST were significantly reduced by the treatment with both CUR and SIL alone or combined, while the levels of ALP were reduced by both compounds in mice exposed to $FB_1$,

and by SIL or SIL and CUR combined in mice exposed to OTA. The kidney injury was also evaluated with the serological measure of creatinine and urea. Creatinine is commonly used as a renal function measure and is the first step in controlling the glomerular filtration rate (GFR). We observed an increase in creatinine levels in mice exposed to OTA and $FB_1$, which was completely neutralized by all treatments in animals exposed to $FB_1$ and by the treatment of CUR and SIL alone in animals exposed to OTA. CUR and SIL combined were able to partially neutralize the creatinine levels increase. The increase in creatinine observed in mice treated with the mycotoxins is related to the GFR reduction, which can be associated with oxygen free radical formation. Actually, oxidative stress can promote the formation of a variety of vasoactive mediators that can affect renal functions directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient and, thus, reduce GFR (Damiano et al., 2020; Papinska and Rodgers, 2018). Our results corroborate a recent study from Damiano et al. (2020), which demonstrated that CUR can prevent renal injury caused by OTA by mitigating the creatinine levels increase caused by the mycotoxin. As for urea, our revealed an increase only in mice exposed to OTA, which were neutralized by the treatments.

The potential ability of CUR and SIL in protecting liver and kidney cells has been reported in *in vitro* (Ledur and Santurio, 2020; Wang et al., 2018; Yu et al., 2018) and *in vivo* studies (Poapolathep et al., 2015; Rastogi et al., 2000; Sozmen et al., 2014). Besides, CUR has shown the ability to counter the oxidative stress caused by zearalenone in porcine granulosa cells in an *in vitro* study by modulating the gene expression and decreasing free radicals in cells (Qin et al., 2015) and several studies reported the protective effects of CUR in different animal models exposed to aflatoxin $B_1$, revealing the hepatoprotective effect of this phytoalexin in the hepatic injury caused by $AFB_1$ (El-Agamy, 2010; El-Bahr, 2015; Poapolathep et al., 2015). As to silymarin, *in vivo* studies demonstrated the ability of this

compound to prevent liver damage caused by mycotoxins in poultry and rodents. In BALB/c mice receiving $FB_1$ contaminated feed, SIL was able to mitigate liver damage (He et al., 2004; Sozmen et al., 2014), while in rats, SIL protected liver cells from damage caused by $AFB_1$ by improving the membrane transport, the electron transport chain, gene transcription and oxidative stress (Rastogi et al., 2000). In the same manner, in chicken, SIL was effective in protecting against liver damage caused by $AFB_1$ (Grizzle et al., 2009) and against immunotoxic effects caused by OTA (Khatoon et al., 2013). A recent study also demonstrated that CUR protects rats exposed to OTA from the nephrotoxicity caused by this mycotoxins due to the antioxidant and immunoprotective activity (Damiano et al., 2020).

In conclusion, our findings demonstrate that curcumin and silymarin are both able to protect the liver and the kidneys of mice exposed to OTA and $FB_1$, indicating nephroprotective and hepatoprotective effects due to their antioxidant properties. CUR and SIL, alone or in a co-treatment, showed an improvement in liver and kidney function, which was demonstrated by biochemical and antioxidant enzyme activities and mitigation of oxidative stress in animals exposed to the mycotoxins. These observations were also supported by morphologic results such as reduction in tubular damage and hepatocytes necrosis. Thus, although additional studies concerning the practical application of CUR and SIL are necessary, our results demonstrate that adding these phytochemicals to feed could reduce animal intoxication by mycotoxins, improving animal and human health.

**Conflict of interest statement**

There are no conflicts of interests to declare in this study.

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# 6 DISCUSSÃO

As micotoxinas são um importante grupo de contaminantes de alimentos que causam uma série de efeitos deletérios em quem as consome, sendo de fundamental importância compreender seus mecanismos de toxicidade, bem como buscar alternativas que minimizem esses efeitos, uma vez que por mais que se tenha cuidado em todas as etapas de produção e armazenamento de cereais e outros alimentos que podem ser contaminados com micotoxinas, é muito difícil eliminar totalmente os fungos de todas as etapas de produção.

Nesse estudo, foram investigados inicialmente os efeitos tóxicos da ocratoxina A (OTA) em dois tipos de células de suínos, as células renais da linhagem PK-15, e células mononucleares do sangue periférico (PBMCs). A nefrotoxicidade causada pela OTA é significativamente dependente da espécie, principalmente devido às diferenças na meia-vida de eliminação da OTA, que varia significativamente entre as diferentes espécies. Dessa forma, para este estudo escolhemos células suínas devido à alta suscetibilidade dos suínos aos efeitos tóxicos da OTA e sua importância na produção animal. Nossos resultados mostraram um aumento nos níveis de espécies reativas de oxigênio (EROs) tanto em células PK-15 como em PBMCs expostas à OTA. Além disso, observamos um aumento na formação de malondialdeído (MDA) em decorrência da peroxidação lipídica de células tratadas com diferentes concentrações de OTA.

Com base nas evidências atualmente disponíveis, acredita-se que o estresse oxidativo seja a causa da toxicidade induzida pela OTA, porém os mecanismos ainda não estão completamente compreendidos (DA SILVA et al., 2018). Nesse sentido, o estresse oxidativo é causado por um desequilíbrio entre a manifestação sistêmica de radicais livres e a capacidade do sistema biológico de prontamente inativar os intermediários reativos ou reparar o dano resultante (SCHIEBER; CHANDEL, 2014).

Por esse motivo, foi investigado o efeitos da OTA sobre a atividade das enzimas superóxido dismutase (SOD) e catalase (CAT), que são enzimas-chave do sistema antioxidante endógeno mitocondrial. Foi observado que a OTA aumentou significativamente a atividade total da SOD em baixas concentrações, o que pode indicar um esforço para manter o equilíbrio no estado redox celular, evitando o dano oxidativo. Apesar de ser uma enzima antioxidante, um aumento da atividade da SOD pode induzir o estresse oxidativo devido ao acúmulo de peróxido. Isso ocorre quando o aumento da atividade da SOD não é acompanhado pelo aumento da atividade das enzimas removedoras de peróxido de hidrogênio e é caracterizado por uma razão SOD/CAT aumentada (PINHO et al., 2006). Nossos resultados

mostraram uma inibição da atividade da CAT em baixas concentrações de OTA, acima de 1 µM, indicando um desequilíbrio entre a razão SOD/CAT. Nas concentrações intermediárias de OTA, não foi observada mudança significativa na atividade total da SOD, e em concentrações acima de 10 µM a atividade de SOD foi significativamente reduzida. Além disso, nessas concentrações o dano oxidativo aos lipídeos já foi observado por meio do ensaio TBARS. Esses resultados estão de acordo com um estudo que mostrou que a exposição à OTA diminui a atividade de SOD em concentrações acima de 10 µM em células tubulares de rim suíno (LLC-PK1). Além disso, os autores também encontraram uma diminuição na CAT, glutationa-S-transferase e glutationa-peroxidase em células LLC-PK1 expostas a concentrações de OTA acima de 10 µM (BOESCH-SAADATMANDI et al., 2008).

Além disso, nossos resultados mostraram que a exposição à OTA aumentou significativamente a fosforilação oxidativa, indicando uma tentativa celular de compensar a depleção de ATP causada pela forte inibição do complexo I da cadeia transportadora de elétrons. Estes resultados estão de acordo com estudos anteriores, que demonstraram que a OTA exerce seu efeito na respiração mitocondrial e na fosforilação oxidativa por meio do comprometimento da membrana mitocondrial e da inibição das atividades de transferência de elétrons suportadas por succinato da cadeia respiratória (LI et al., 2019).

Com base nessas evidências e nos resultados obtidos com células renais PK-15 e PBMCs de suínos, podemos entender que os efeitos tóxicos da OTA são consequência do desequilíbrio do sistema redox das células e consequente produção de EROs. A partir disso, levando em conta esse mecanismo de toxicidade, podemos buscar alternativas que minimizem ou neutralizem os danos dessa micotoxina. Nesse contexto, seguimos este estudo com objetivo determinar se a curcumina e a silimarina poderiam reduzir ou mesmo prevenir os danos induzidos pelas micotoxinas OTA, $FB_1$ e DON em células renais de suínos e para isso, as células da linhagem PK-15 foram pré-tratadas com estes compostos e posteriormente expostas às micotoxinas, para avaliação de viabilidade celular, produção de EROs e apoptose.

Os resultados demonstraram que o pré-tratamento durante 6 h com curcumina e silimarina evitou a perda de viabilidade celular nas células renais expostas à OTA por 48 h, no entanto, nenhuma das concentrações testadas *in vitro* foi capaz de neutralizar totalmente a toxicidade da OTA. Por outro lado, nenhum dos tratamentos reduziu a produção de EROs ou preveniu a apoptose celular induzida pela exposição OTA após 48 horas de tratamento. Resultados semelhantes foram encontrados em um estudo que investigou os efeitos protetores do resveratrol em células renais embrionárias humanas (HEK293) expostas a OTA, onde as EROs intracelulares foram significativamente aumentadas após 48 h de tratamento, no

entanto, outros parâmetros como perda de viabilidade celular e genotoxicidade por quebras de DNA de fita simples foram significativamente reduzidas (RAGHUBEER et al., 2015). O rim é o principal alvo da toxicidade da OTA devido ao seu papel na excreção desta micotoxina pela urina, permitindo que a toxina exerça seus efeitos tóxicos nos túbulos renais e comprometendo a função renal (KOSZEGI; POOR, 2016), o que poderia explicar uma grande dificuldade de proteção células renais da toxicidade de OTA *in vitro*.

Quando expostas a $FB_1$, as células PK-15 pré-tratadas com CUR apresentaram um aumento na viabilidade celular e uma inibição significativa na produção de EROs, enquanto nenhuma redução significativa na taxa de apoptose foi observada. Por outro lado, o SIL foi capaz de prevenir totalmente a perda de viabilidade celular e indução de apoptose e reduzir significativamente a produção de ROS. A indução de apoptose da $FB_1$ se dá por meio da inibição da enzima ceramida sintase, levando ao acúmulo intracelular de esfingolipídios que são pró-apoptóticos, inibidores de crescimento citotóxicos e imunotóxicos (MARASAS et al., 2004) e também por geração de EROs (DOMIJAN et al., 2015; HASSAN et al., 2015; ABBES et al., 2016). Portanto, o efeito antiapoptótico apresentado pelo SIL nas células PK-15 expostas à $FB_1$ pode ser decorrente de sua imunoproteção além de sua atividade antioxidante, visto que há relatos de que o SIL inibe também os efeitos imunotóxicos causados pelas micotoxinas (KHATOON et al., 2013). No entanto, ainda não está claro se o estresse oxidativo emerge como uma consequência ou como um mecanismo causal da toxicidade de $FB_1$ (WANG et al., 2016; DA SILVA et al., 2018).

Já nas células expostas ao DON, o pré-tratamento com CUR e SIL foi capaz de proteger completamente as células contra a perda de viabilidade celular e produção de EROs e também reduziu a taxa de apoptose. Estes resultados estão de acordo com estudo realizado em ratos, que mostrou que nanopartículas de silimarina foram capazes de proteger o fígado destes animais contra a hepatotoxicidade induzida por DON por meio da modulação de enzimas antioxidantes e diminuição da genotoxicidade (ABDEL-WAHHAB et al., 2018).

A partir desse resultado positivo *in vitro*, tornou-se relevante investigar o potencial da curcumina e da silimarina como possíveis aditivos alimentares e para isso foi realizado o experimento *in vivo* em camundongos, avaliando o efeito protetor destes compostos frente à toxicidade aguda induzida por OTA e $FB_1$. Para isso, foram avaliados parâmetros de estresse oxidativo nos rins e fígado de animais pré-tratados com curcumina e/ou silimarina e expostos às micotoxinas, além de determinação de marcadores bioquímicos de função hepática como a aspartato aminotransferase (AST), a alanina aminotransferase (ALT) e fosfatase alcalina

(ALP), e de função renal, como a creatinina e a ureia, no soro desses animais, além da investigação de alterações histológicas.

Tanto OTA quanto $FB_1$ causaram um aumento nos parâmetros de estresse oxidativo no fígado de camundongos. Nossos dados mostraram um aumento na formação de MDA e sua consequente peroxidação lipídica, um aumento na carbonilação de proteínas, indicando danos à proteína e também um aumento na formação de EROs. No entanto, tanto CUR como SIL foram capazes de mitigar esses efeitos. Em geral, CUR e SIL desempenharam um papel semelhante na proteção do fígado contra a toxicidade das micotoxinas, porém os níveis mais baixos de MDA foram alcançados no fígado de camundongos tratados apenas com SIL, mitigando completamente a peroxidação lipídica, indicando que SIL poderia oferecer uma melhor proteção contra lesão hepática induzida por micotoxinas. Um estudo anterior demonstrou que os pré-tratamentos com SIL impediram a formação de MDA e as células protegidas do estresse oxidativo por meio da regulação positiva de enzimas do sistema antioxidante em hepatócitos expostos à OTA (YU et al., 2018).

O rim é o principal órgão alvo da OTA e a nefrotoxicidade também foi relacionada como consequência da intoxicação por $FB_1$ (KOSZEGI; POOR, 2016; DA SILVA et al., 2018). Neste estudo, foi observado um aumento em todos os parâmetros oxidativos testados no rim de camundongos expostos a OTA e $FB_1$. Embora a SIL não tenha sido capaz de reduzir os níveis de ROS no rim de animais expostos a FB1, foi capaz de reduzir parcialmente os níveis de ROS em camundongos expostos à OTA, enquanto o CUR atenuou completamente a formação de ROS em animais expostos a ambas as micotoxinas. Por outro lado, CUR e SIL foram capazes de mitigar o a formação de MDA e a carbonilação de proteínas. Estes dados confirmam a hipótese apontada no estudo *in vitro* (protocolo experimental II), de que CUR e SIL teriam um grande potencial na proteção renal de animais expostos às micotoxinas. Além disso, recentemente foi relatado o efeito protetor de CUR em danos renais induzidos por OTA e o a atividade antioxidante de CUR foi apontada como responsável por esses efeitos (DAMIANO et al., 2020).

Ainda dentro da hipótese oxidativa, foi realizada a determinação da atividade da enzima antioxidante mitocondrial catalase (CAT), onde observamos uma inibição da atividade da CAT no fígado de camundongos expostos a OTA e nos rins de camundongos expostos a OTA e $FB_1$. Esta inibição da CAT em animais expostos à OTA foi neutralizada pelo tratamento com SIL e também por CUR e SIL combinadas. Já nos rins de animais expostos à $FB_1$, apenas o tratamento com a combinação de CUR e SIL foi capaz de aumentar a atividade da CAT para os níveis normais. Estes resultados são corroborados por estudos que mostram

que SIL e CUR foram capazes de proteger o fígado contra a hepatotoxicidade induzida por diferentes micotoxinas por meio da modulação de enzimas antioxidantes e diminuição da genotoxicidade (EL-BAHR, 2015; ABDEL-WAHHAB et al., 2018; RUAN et al., 2019).

Os achados histológicos observados no estudo *in vivo* revelaram necrose tubular moderada e congestão moderada nos rins de camundongos expostos à OTA e $FB_1$. Além disso, células necróticas foram observadas no fígado de animais expostos ambas micotoxinas. A curcumina e a silimarina, isoladamente ou combinadas, diminuíram o aparecimento de células necróticas no fígado, enquanto que nos rins, a curcumina e a silimarina combinadas não impediram o dano histológico em camundongos expostos às micotoxinas. Achados semelhantes foram observados por DAMIANO et al. (2020) em um estudo que relatou que o tratamento com CUR apresentou redução no número de necrose glomerular e tubular. Foi relatado que SIL diminuiu significativamente o número de hepatócitos apoptóticos no fígado de camundongos expostos a $FB_1$ e a regeneração celular foi observada enquanto diminuía o dano celular (HE et al., 2004; SOZMEN et al., 2014). Além da atividade antioxidante observada neste estudo, esse efeito protetor da silimarina também pode ser explicado pelo efeito estimulador da silimarina na regeneração de tecidos hepáticos lesados (HE et al., 2004).

Com relação a determinação de marcadores bioquímicos de função hepática, os resultados obtidos estão de acordo com outros estudos (HE et al., 2004; YU et al., 2018; DAMIANO et al., 2020). Foi observado aumento significativo na atividade de ALT, AST e ALP no soro de animais expostos a OTA e $FB_1$, com níveis maiores observados com a exposição à $FB_1$ do que à OTA, indicando um maior dano hepático induzido por essa micotoxina. No entanto, os níveis de ALT e AST foram significativamente reduzidos pelo tratamento com CUR e SIL, administradas isoladamente ou combinadas, enquanto os níveis de ALP foram reduzidos por ambos os compostos em camundongos expostos a $FB_1$, e por SIL ou SIL e CUR combinados em camundongos expostos para OTA.

A lesão renal também foi avaliada com a dosagem de creatinina e uréia. Foi observado um aumento nos níveis de creatinina em camundongos expostos a OTA e $FB_1$, que foi completamente neutralizado por todos os tratamentos em animais expostos a $FB_1$ e pelo tratamento de CUR e SIL isoladamente em animais expostos a OTA. O aumento da creatinina observado em camundongos tratados com micotoxinas está relacionado à redução da taxa de filtração glomerular, que pode estar associada à formação de EROs. O estresse oxidativo pode promover a formação de uma variedade de mediadores vasoativos que podem afetar as funções renais diretamente, causando vasoconstrição renal ou diminuindo o coeficiente de ultrafiltração capilar glomerular e, assim, reduzir a taxa de filtração glomerular (PAPINSKA;

RODGERS, 2018; DAMIANO et al., 2020). Nossos resultados são corroborados um estudo recente de DAMIANO et al. (2020), que demonstrou que a CUR pode prevenir a lesão renal causada pela OTA em ratos, ao mitigar o aumento dos níveis de creatinina. Quanto à uréia, foi observado aumento apenas em camundongos expostos à OTA, que foram neutralizados pelos tratamentos.

A curcumina e a silimarina são duas fitoalexinas com conhecida atividade antioxidante que vêm sendo estudadas para diversos fins terapêuticos. Além disso, existem relatos na literatura que apontam para os efeitos protetores desses compostos contra efeitos causados por intoxicação por micotoxinas diversas e também outras toxinas e produtos tóxicos. A curcumina demonstrou a capacidade de neutralizar o estresse oxidativo causado pela zearalenona em células da granulosa suína em um estudo *in vitro* (QIN et al., 2015) e também foi capaz de diminuir a hepatotoxicidade e hepatocarcinogenicidade induzida por aflatoxina $B_1$ ($AFB_1$) em ratos por meio da modulação de enzimas antioxidantes (EL-AGAMY, 2010; EL-BAHR, 2015; POAPOLATHEP et al., 2015). Quanto à silimarina, estudos demonstraram a capacidade dessa fitoalexina em prevenir danos ao fígado causados por micotoxinas em aves e roedores. Em camundongos BALB/c recebendo ração contaminada com $FB_1$, a silimarina foi capaz de reverter os danos ao fígado causados por esta micotoxina (HE et al., 2004; SOZMEN et al., 2014) e em ratos, a silimarina foi capaz de proteger as células do fígado dos danos causados por $AFB_1$, melhorando o transporte da membrana, a cadeia de transporte de elétrons, a transcrição gênica e o estresse oxidativo (RASTOGI et al., 2000).

Em geral, tomando em conjunto os resultados obtidos neste estudo, pode-se afirmar que estes resultados reforçam o papel fundamental do estresse oxidativo como mecanismo de toxicidade da OTA e também da $FB_1$, e demonstram que a curcumina e a silimarina apresentam efeitos nefro e hepatoprotetores em camundongos expostos à diferentes micotoxinas. Dessa forma, apesar de ser necessário um estudo da aplicabilidade prática e estudos clínicos mais amplos, é possível concluir que a curcumina e a silimarina podem ser utilizadas como aditivo alimentar em rações de animais a fim de promover proteção contra efeitos deletérios provocados pela exposição à micotoxinas, melhorando a saúde animal e humana.

## 7 CONCLUSÃO

De maneira geral, os resultados obtidos neste estudo sugerem que:

- A ocratoxina A causa diminuição na viabilidade celular, aumento no estresse oxidativo e peroxidação lipídica em células de epitélio renal suíno da linhagem PK-15 e em células mononucleares de sangue periférico de suínos, além de causar um desequilíbrio na atividade da enzima superóxido dismutase e inibição na atividade da catalase. Além disso, foi observado que OTA é um inibidor do complexo I da cadeia transportadora de elétrons, causando danos ao funcionamento mitocondrial. Os danos provocados pela OTA em células renais da linhagem PK-15 são semelhantes aos causados em PBMCs de suínos no que se refere a perda de viabilidade celular e estresse oxidativo.

- Tanto o pré-tratamento com curcumina quanto com silimarina são capazes de reduzir a toxicidade induzida pelas micotoxinas ocratoxina A, fumonisina $B_1$ e deoxinivalenol *in vitro* sobre células da linhagem PK-15. O pré-tratamento com curcumina diminuiu a perda de viabilidade das células expostas às micotoxinas, diminuiu a formação de espécies reativas de oxigênio (EROs) e reduziu a apoptose causada por DON. Já o pré-tratamento com silimarina reduziu a perda de viabilidade em células expostas à OTA e neutralizou a perda de viabilidade induzida por FB1 e DON e também diminuiu a formação de EROs e apoptose induzidas por $FB_1$ e DON.

- Em camundongos, o pré-tratamento com curcumina e silimarina tanto isoladas quanto em combinação mostraram efeitos hepato e nefroprotetores sobre a toxicidade aguda induzida por OTA e $FB_1$. Os resultados indicam que este efeito protetor está relacionado às propriedades antioxidantes destas fitoalexinas. Curcumina e silimarina determinaram uma melhora na função hepática e renal em animais expostos às micotoxinas, o que foi demonstrado pela diminuição de marcadores enzimáticos no soro dos animais e pela diminuição do estresse oxidativo desencadeado por OTA e $FB_1$. Essas observações foram apoiadas por resultados morfológicos, como redução do dano tubular e necrose de hepatócitos. Este conjunto de resultados demonstra que a adição de curcumina e/ou silimarina à ração pode reduzir a intoxicação animal por micotoxinas, melhorando a saúde animal.

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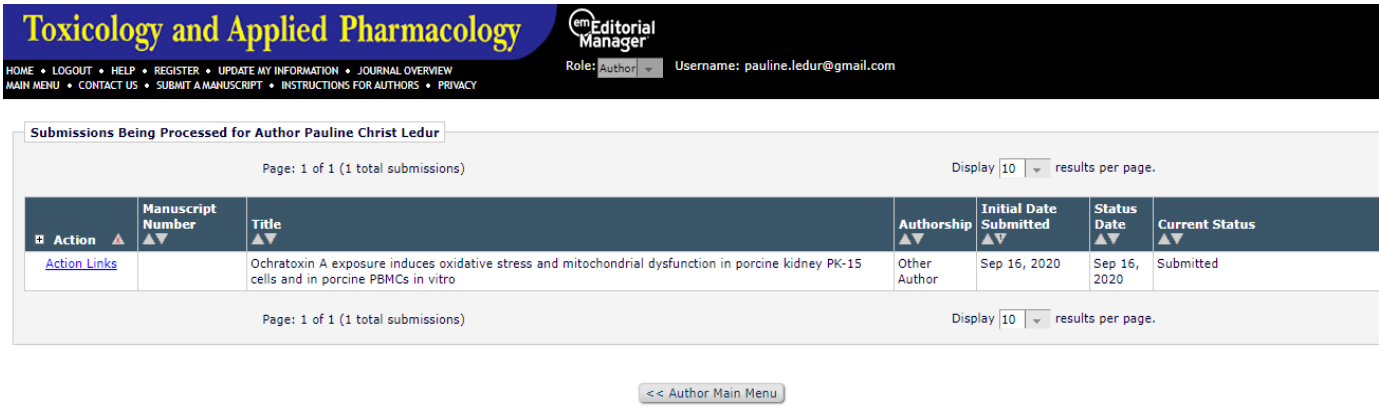
# ANEXO A – COMPROVANTE DE SUBMISSÃO DO MANUSCRITO DO PROTOCOLO EXPERIMENTAL I



# ANEXO B – CERTIFICADO DA CEUA PARA O PROTOCOLO EXPERIMENTAL I

*Comissão de Ética no Uso de Animais*
*da*
*Universidade Federal de Santa Maria*

## CERTIFICADO

Certificamos que a proposta intitulada "Machos suínos reprodutores alimentados com dietas comerciais enriquecidas com extratos vegetais: características do ejaculado e dose inseminante", protocolada sob o CEUA nº 7311050517, sob a responsabilidade de **Carlos Augusto Rigon Rossi** *e equipe; Maurício da Cruz Franco; Alexia Pretto; Cristian Guilherme Gräf; Julianni Dornelles; Luara Medianeira Schlösser; Marcelo Soares; Nicolas Carmo de Avila* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 07/06/2017.

We certify that the proposal "Boar fed commercial diets enriched with plant extracts: characteristics of the ejaculate and inseminating dose", utilizing 4 Swines (4 males), protocol number CEUA 7311050517, under the responsibility of **Carlos Augusto Rigon Rossi** *and team; Maurício da Cruz Franco; Alexia Pretto; Cristian Guilherme Gräf; Julianni Dornelles; Luara Medianeira Schlösser; Marcelo Soares; Nicolas Carmo de Avila* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 06/07/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2017 a 12/2017 Área: Clínica de Grande Animais

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Origem: | Biotério externo | | | | | | |
| Espécie: | Suínos | sexo: | Machos | idade: | 2 a 3 anos | N: | 4 |
| Linhagem: | MS 115 - Linhagem EMBRAPA | | | Peso: | 250 a 300 kg | | |

Resumo: O estudo objetiva avaliar as características seminais de machos suínos reprodutores alimentados com dietas comerciais enriquecidas com extratos vegetais (EV) (ácido ascórbico e bioflavonóides). O estudo será realizado em duas etapas: Etapa 1- Utilizar-se-ão quatro reprodutores híbridos com 550 dias de idade e 280 ± 12,0kg distribuídos em quatro tratamentos: (T1): dieta Controle (C) (ração comercial para machos reprodutores suínos); T2: C+300g de EV; T3: C+600g de EV; T4: C+900g de EV. Neste período (45 dias), os machos receberam a dieta enriquecida com EV (período de adaptação ao EV). O estudo transcorrerá por mais 45 dias (para avaliar as possíveis respostas ao uso dos EV), totalizando 90 dias experimentais. O sêmen será coletado dos quatro machos suínos, uma vez por semana, durante o período de 45 dias. Nessa etapa, serão avaliadas as características seminais (vigor espermático (0 a 5), motilidade espermática (%), concentração (x106sptz mL-1) e morfologia (%) (problemas de cabeça, cauda, peça intermediária, presença de gota citoplasmática proximal e os espermatozoides serão classificados em normais e/ou danificados) e integridade de membrana espermática. Os animais serão submetidos a coletas de sangue (veia femoral, simultaneamente a coleta de sêmen) nos dias 0, 15, 30 e 45 de cada período experimental. O sangue coletado será utilizado para o hemograma e dosagem bioquímica das enzimas ALT, AST, CK e Creatinina. Amostras de sangue serão acondicionadas em tubos de coleta sanguínea para dosagem dos níveis de testosterona e cortisol. Após a etapa 1, os animais permanecerão em descanso por 45 dias para iniciar a etapa 2, a qual segue o mesmo protocolo da etapa 1, diferenciando pelo □cruzamento□ dos tratamentos, para remoção do efeito individual do macho. O delineamento experimental utilizado será em quadrado latino 4x4 (quatro animais, quatro tratamentos, quatro períodos e quatro coletas de sangue por período). A análise das diferenças entre médias será feita por variância multifatorial usando-se o General Linear Models do MINITAB/2010, com significância P<0,05. A expectativa do estudo será que haverá diferenças quanto à motilidade, concentração espermática quantificadas pela câmara de Neubauer e alterações morfológicas para os animais que receberão dietas enriquecidas com EV. As percentagens de células com plasma e membranas íntegras, peroxidação lipídica da membrana acrossomal e potencial de membrana mitocondrial não serão alterados nos animais que receberão dietas enriquecidas com EV. A utilização de EV na dieta dos machos reprodutores suínos pode, individualmente e em combinação, melhorar a capacidade e características espermáticas, devido seu efeito sobre a diminuição da produção de espécies de oxigênio reativos.

Local do experimento: Laboratório de Andrologia Veterinária-Androlab

Santa Maria, 07 de junho de 2017

Avenida Roraima, 1000, Reitoria, 2º andar - CEP 97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax:
Horário de atendimento: das 8:30 ás 12h e 14h ás 17hs : e-mail: ceua.ufsm@gmail.com
CEUA N 7311050517

*Comissão de Ética no Uso de Animais*
*da*
*Universidade Federal de Santa Maria*

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

# ANEXO C – ARTIGO PUBLICADO

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## Cytoprotective effects of curcumin and silymarin on PK-15 cells exposed to ochratoxin A, fumonisin $B_1$ and deoxynivalenol

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ARTICLE INFO

*Keywords:*
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Curcumin
Silymarin

ABSTRACT

Mycotoxins are toxic secondary metabolites produced by fungus which cause worldwide concern regarding food and feed safety. Ochratoxin A (OTA), fumonisin $B_1$ ($FB_1$) and deoxynivalenol (DON) are some of the main mycotoxins and oxidative stress is the main mechanism of toxicity. Thereby, this study investigates the in vitro cytoprotective effects of curcumin (CUR) and silymarin (SIL) - known for their strong antioxidant activity - in PK-15 cells exposed to OTA, $FB_1$ and DON. Pretreatment with CUR and SIL enhanced the viability of cells exposed to the mycotoxins ($P < 0.001$) and attenuated reactive oxygen species (ROS) formation by DON ($P < 0.01$), partially reduced ROS formation by $FB_1$ ($P < 0.001$), but not OTA. CUR significantly decreased apoptosis in cells exposed to DON ($P < 0.01$) but was not able to prevent apoptosis in cells exposed to OTA and $FB_1$. Whereas SIL was able to prevent apoptosis in PK-15 cells exposed to $FB_1$ and DON ($P < 0.01$) but was not able to decrease apoptosis in cells exposed to OTA. In summary, these data indicate that curcumin and silymarin are able to provide cytoprotection against toxicity induced by OTA, $FB_1$ and DON in PK-15 cells.

### 1. Introduction

Mycotoxins are second metabolites produced by filamentous fungi that are toxic even in low concentrations to vertebrates and other groups of animals. Moreover, mycotoxins are the most commonly occurring natural food contaminant in human and animal feed. (Bennett and Klich, 2003; Marroquin-Cardona et al., 2014). The animal production industry is most commonly affected by mycotoxins. Overall, most mycotoxins cause immunosuppression which leaves animals more vulnerable to diseases by weakening their immune system or making them less responsive to vaccinations. In acute cases, losses are related to mortality. Other subclinical effects may cause loss in productivity, reduce weight gains and interfere in feed efficiency (Marroquin-Cardona et al., 2014).

Ochratoxin A (OTA), fumonisin $B_1$ ($FB_1$) and deoxynivalenol (DON) are some of the most common mycotoxins, produced by different fungus of genera *Aspergillus*, *Penicillium* and *Fusarium* (Marin et al., 2013). OTA can be found in contaminated food grains and cereals, fruit, fruit products and coffee. The main mechanism of OTA toxicity consists of high production of reactive oxygen species (ROS), which leads to mitochondrial dysfunction, cellular damage and genotoxicity. The kidney is the organ most affected by OTA toxic effects due to its role to the mycotoxin excretion through the urine, allowing the toxin to exert its toxic effects on kidney tubules, impairing kidney function (Koszegi and Poor, 2016; Raghubeer et al., 2017). Fumonisins are mostly found in maize which are contaminated with *Fusarium verticillioides* and *Fusarium proliferatum*. $FB_1$ is the most important fumonisin from a toxicological perspective and its toxicity mechanism is based on the fact that $FB_1$ has a structure similar to sphingoid bases such as sphingosine, and can function as an inhibitor of ceramide synthase, therefore, this mycotoxin strongly inhibits the enzyme ceramide synthase that catalyzes the acylation of sphinganine and recycling of sphingosine. The inhibition of CER synthase increases intracellular sphinganine and other sphingoid bases, which are highly cytotoxic compounds (Liu et al., 2019). Other studies have demonstrated the potential of $FB_1$ to promote oxidative stress with consequent increase in intracellular ROS levels and their cytotoxic effects, and induction of apoptosis (Abbes et al., 2016; Domijan et al., 2015), $FB_1$ is also able to modulate the expression of enzymes of the antioxidant system and to increase MDA levels (Abbes et al., 2016; da Silva et al., 2018; Domijan et al., 2007). DON is a type B trichothecene predominantly produced by *Fusarium graminearum* and *Fusarium culmorum* (Bennett and Klich, 2003). Ingestion of DON contaminated foods induces vomiting, anorexia, disturbance of cell

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# ANEXO D – CERTIFICADO DA CEUA REFERENTE AO PROTOCOLO EXPERIMENTAL III

*Comissão de Ética no Uso de Animais*
*da*
*Universidade Federal de Santa Maria*

Santa Maria, 26 de março de 2019
CEUA N 1185101117

IImo(a). Sr(a).
Responsável: Janio Morais Santurio
Área: Microbiologia E Parasitologia
Janio Morais Santurio (orientador)

Título da proposta: "Avaliação dos efeitos de curcumina e silimarina sobre a toxicidade induzida por fumonisina B1 e ocratoxina A in vitro e in vivo".

**Parecer Consubstanciado da Comissão de Ética no Uso de Animais UFSM**

A Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 28/fevereiro/2019) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Esta emenda propõe o uso de camundongos como modelo experimental para possibilitar a finalização das atividades experimentais de projeto de Doutorado em Farmacologia conforme projeto original aprovado pelo GAP, uma vez que os resultados obtidos nos testes in vitro demonstraram o potencial da curcumina e da silimarina na proteção das células diante da toxicidade causada pelas micotoxinas Ocratoxina A e Fumonisina B1, conforme detalhado no arquivo da justificativa em anexo. Os camundongos são um dos modelos animais mais utilizados na experimentação em testes de toxicidade. Vários estudos demonstram que camundongos são um bom modelo para avaliar a neutralização de efeitos tóxicos causados por toxinas (Parasuraman, 2011). Em estudos prévios com camundongos, foi demonstrado que a silimarina tem potencial para neutralizar danos hepáticos causados pela fumonisina B1 (HE et al., 2004; SOZMEN et al., 2014), bem como para demonstrar os efeitos tóxicos e os mecanismos de toxicidade da ocratoxina A (BONDY et al., 2015; KURODA et al., 2015). Também já foram demonstrados efeitos protetores da curcumina frente à toxicidade causada pela Aflatoxina B1 (OAPOLATHEP et al., 2015). Além disso, destaca-se a necessidade de se realizarem testes in vivo em camundongos, uma vez que permitem a avaliação dos parâmetros toxicológicos do organismo como um todo, e não apenas de um tipo de células isoladas, o que é fundamental para o estudo da interação das diferentes substâncias testadas sobre o organismo. O delineamento experimental, bem como todas as atividades propostas nesta emenda encontram-se em anexo.".

Comentário da CEUA: "Emenda aprovada nos seus aspectos éticos.".

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