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JULIANA RUBIRA GEREZ

EFEITOS REPRODUTIVOS E SISTÊMICOS DE DESOXINIVALENOL E FUMONISINA B1 EM MODELO *EX VIVO* E *IN VIVO* EM RATOS E SUÍNOS

Londrina
2022

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Tese apresentada à banca de defesa do Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina como requisito para obtenção do título de Doutora em Ciências da Saúde.

Orientador: Prof Dr Waldiceu Aparecido Verri Júnior
Co-orientadora: Profª Drª Ana Paula F.R.L. Bracarense

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Londrina, 26 de maio de 2022.

Àos meus pais, que tanto lutaram por minha educação.

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“Quando a educação não é libertadora, o sonho do oprimido é ser opressor”.

Paulo Freire

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RESUMO

Micotoxinas são toxinas fúngicas, encontradas em alimentos a base de cereais no mundo todo. Desoxinivalenol (DON) e fumonisina B1 (FB1) são as mais frequentes fusariotoxinas e induzem toxicidade imune e intestinal em humanos e animais. Recentemente, uma associação entre micotoxinas e alterações na fertilidade tem sido sugerida, mas são escassos os estudos in vivo relacionados aos efeitos de micotoxinas sobre o sistema reprodutivo. Ainda, fatores relacionados ao hospedeiro como sexo e idade podem influenciar a toxicidade de DON. Apesar disso, em estudos toxicológicos envolvendo DON, a resposta dependente de sexo e idade tem sido frequentemente negligenciada. Desse modo, o presente trabalho teve como objetivo avaliar a toxicidade ex vivo e in vivo de duas importantes micotoxinas nos ovários de suínos (DON e/ou FB1), testículo e ovários de ratos durante o período juvenil e peripuberal (DON). Em adição, nos ratos o dimorfismo sexual foi avaliado sobre a toxicidade sistêmica de DON, com ênfase na participação do estresse oxidativo no mecanismo toxicológico de DON. Para isso, foram realizados dois experimentos. Experimento 1: Setenta e dois explantes ovarianos foram obtidos de 6 suínos e submetidos aos tratamentos controle (meio MEM), DON (10 µM), FB1 (100 µM) e DON+FB1 (10 µM e 100 µM). Avaliação histológica e imunohistoquímica foram realizadas para identificar alterações histológicas, proliferação celular e apoptose. A peroxidação lipídica e capacidade antioxidante também foram avaliadas. Experimento 2: 24 ratos Wistar juvenis com 28 dias de vida (12 fêmeas e 12 machos) foram divididos em grupo controle (n=12 – 6 fêmeas e 6 machos, dieta livre de micotoxina) e grupo DON (n=12 – 6 fêmeas e 6 machos, dieta contaminada com 10 mg de DON/kg de ração). Após 28 dias de tratamento, amostras de sangue, intestino, fígado, rim, ovário e testículo foram colhidos para análises clínica (bioquímica e hematológica), histopatológica, histoquímica, imunohistoquímica e estresse oxidativo. No experimento 1 a exposição às micotoxinas induziram um aumento de folículos degenerados com uma diminuição de folículos viáveis. Uma diminuiçao significativa na proliferação de células da granulosa foi observada em todos os tratamentos. O tratamento DON+FB1 foi responsável por um aumento no índice apoptótico de folículos em crescimento. Por outro lado, o tratamento com FB1 e combinado induziram uma diminuição na peroxidação lipídica e um aumento na resposta antioxidante. No experimento 2 a toxicidade de DON foi verificada no testículo, ovários e útero de ratos durante o período juvenil e peripuberal. Nos ovários e útero uma redução no peso dos órgãos foi acompanhada por um aumento significativo na expressão de BAX e caspase-3 e diminuição na expressão de BCL-2 na maioria dos estágios foliculares e corpo lúteo. Nos testículos a toxicidade de DON foi associada com uma redução no número de células de Sertoli e Leydig e no número de túbulos seminíferos no estágio XIV. Um aumento no número de regiões organizadoras de núcleolo em túbulos no estágio I-VI foi observada nos animais expostos a micotoxina. Efeitos relacionados ao sexo foram observados na toxicidade sistêmica de DON em ratos submetidos a uma dieta contaminada durante o periodo juvenil e peripuberal. DON induziu resposta anoréxica em machos mas não em fêmeas, enquanto somente fêmeas mostraram um aumento nos níveis de creatinina e triglicerídeos. Independentemente do sexo, os animais expostos à micotoxina apresentaram aumento no número de linfócitos e leucócitos, redução significativa nos níveis de hemoglobina, hematócrito, volume corpuscular médio, hemoglobina corpuscular média e neutrófilos. Em machos e fêmeas alimentados com DON, lesões histológicas foram observadas no intestino, fígado e rim. Finalmente, um aumento do potencial antioxidade no intestino, fígado e rim foi verificado nos animais alimentados com a dieta contaminada. No entanto, este efeito não foi capaz de prevenir o estresse oxidativo no tecido renal. Desse modo, em modelo ex vivo DON e FB1 sozinhas e em combinação modulam a resposta ao estresse oxidativo, interferindo na produção de radicais livres e afetando a capacidade reprodutiva de suínos. Em modelo in vivo a exposição juvenil e peripuberal ao DON induz a ativação de via apoptótica nos ovários e compromete a estrutura testicular de ratos alterando a dinâmica da espermatogênese. Na exposição juvenil e peripuberal a DON, a predileção de machos aos efeitos anoréxicos de DON foram confirmados e podem influenciar parâmetros bioquímicos. DON induziu alterações hematológicas e histológicas no intestino, fígado e rim em machos e fêmeas. Nos rins de animais expostos a dieta contaminada com a micotoxina, o estresse oxidativo é um importante mecanismo na toxicidade alimentar de DON.

Palavras-chave: desoxinivalenol, fumonisina B1, toxicidade gonadal, dimorfismo sexual, histologia, estresse oxidativo.

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ABSTRACT

Mycotoxins are fungal toxins found in cereal-based foods and feed around the world. Deoxynivalenol (DON) and fumonisin B1 (FB1) are the most frequent fusariotoxins and induce immune and intestinal toxicity in humans and animals. Recently, an association between mycotoxins and changes fertility has been suggested, however in vivo studies related to the effects of mycotoxins on the reproductive system are scarce. In addition, host-related factors such as sex and age can influence the DON toxicity. Despite this, in toxicological studies involving DON, the sex- and the age-dependent response has often been neglected. Thus, the present study aimed to evaluate the ex vivo and in vivo toxicity of two important mycotoxins in the ovaries of pigs (DON and/or FB1), testis, and ovaries of pubertal rats (DON). In addition, in pubescent rats, sexual dimorphism was evaluated on the systemic toxicity of DON, with the emphasis on the participation of oxidative stress in the toxicological mechanism of DON. For this, two experiments were carried out. Experiment 1: Seventy-two ovarian explants were obtained from 6 pigs and subjected to control treatments (MEM medium), DON (10 μM), FB1 (100 μM) and DON and FB1 (10 μM and 100 μM). Histological and immunohistochemical analyses were performed to identify histological changes, cell proliferation, and apoptosis. In addition, lipid peroxidation and antioxidant capacity were evaluated. Experiment 2: 24 juvenil Wistar rats (12 females and 12 males) (28 days old) were divided into a control group (n=12, 6 females and 6 males, mycotoxin-free diet) and DON group (n=12, 6 females and 6 males, diet contaminated with 10 mg DON/kg feed). After 28 days of treatment, blood, intestine, liver, kidney, ovary, and testis samples were collected for clinical, histopathological, histochemical, immunohistochemical, and oxidative stress analysis. In experiment 1, exposure to mycotoxins induced an increase in degenerated follicles with a decrease in viable follicles. A significant decrease in granulosa cell proliferation was observed in all treatments. In addition, the combined treatment was responsible for an increase in the apoptotic index of growing follicles. On the other hand, FB1 and combined treatment induced a decrease in lipid peroxidation and an increase in antioxidant response. In experiment 2, the DON toxicity was verified in the testis, ovaries, and uterus of rats during juvenil and peripubertal periods. In the ovaries and uterus, a reduction in organ weight was accompanied by a significant increase in BAX and caspase-3 expression and a decrease in BCL-2 expression in the majority of follicular stages and corpus luteum. In the testis, DON toxicity was associated with a reduction in the number of Sertoli and Leydig cells and in the number of tubules at stage XIV. An increase in the number of nucleus organizer regions in stage I-VI tubules was observed in animals exposed to the mycotoxin. Sex-related effects were observed on the systemic toxicity of DON in rats during the juvenile and peripubertal periods. DON induced an anorexic response in males but not females, while only females show an increase in creatinine and triglyceride levels. Independently of sex, animals exposed to mycotoxin showed increase in the number of lymphocytes, significant reduction in the levels of hemoglobin, mean corpuscular hemoglobin, leukocytes and neutrophils. In males and females fed the DON-diet, histological changes were observed in the intestine, liver, and kidney. Finally, an increase in the antioxidant potential in the intestine, liver, and kidney was identified in animals DON-diet. However, this effect was not able to prevent oxidative stress in kidney tissue. Thereby, in an ex vivo model DON and FB1 alone and in combination modulate the response to oxidative stress, interfering with the production of free radicals and affecting the reproductive capacity of pigs. In in vivo model, peripubertal exposure to DON induces the activation of the apoptotic pathway in the ovaries and compromises the testicular structure of rats, changing the dynamics of spermatogenesis. At juvenil and peripuberal exposure to DON, male predilection to the anorexic effects of DON has been confirmed and may influence biochemical parameters. DON induced hematological and histological changes in the intestine, liver, and kidney in males and females. In addition to the kidneys of pubertal animals, oxidative stress is an important mechanism in the food toxicity of DON.

Key words: deoxynivalenol; fumonisin B1; gonanal toxicity; sexual dimorphism; histology; oxidative stress.

SUMÁRIO

1. INTRODUÇÃO

A infertilidade é a incapacidade de um casal obter gravidez no período de um ano mesmo tendo relações sexuais sem uso de nenhuma forma de anticoncepção. Esta doença tem sido identificada como um problema crescente de saúde pública afetando em torno de 48,5 milhões de casais no mundo todo (Mascarenhas, Flaxman et al. 2012). Um recente estudo com foco na prevalência de infertilidade mostrou taxas crescentes de 1990 a 2017, afetando 0,37% das mulheres e 0,29% dos homens por ano (Sun, Gong et al. 2019). Em contrapartida numerosas substâncias sintéticas (pesticidas, químicos industriais e bisfenol) e naturais (micotoxinas e fitoestrógenos) têm sido implicadas na desregulação do sistema endócrino, produzindo alterações reprodutivas em humanos e animais (Marques-Pinto ,Carvalho 2013, Demaegdt, Daminet et al. 2016). Micotoxinas como fumonisina B1 (FB_1), zearalenona (ZEA), e tricotecenos tais como desoxinivalenol (DON) produzidos principalmente por fungos do gênero Fusarium são contaminantes de *commodities* agrícolas no mundo todo (Milićević, Škrinjar et al. 2010). O consumo de grãos e alimentos contaminados com fusariotoxinas induzem uma variedade de efeitos adversos e representam um sério risco para a saúde humana e animal (Fink-Grernmels 1999). Embora fusariotoxinas tenham sido consideradas como reguladores potentes da esteroidogênese intracelular, estudos sobre a toxicidade reprodutiva causada por DON e FB_1 em animais são escassos e os dados disponíveis são principalmente baseados em modelos *in vitro* (Cortinovis, Pizzo et al. 2013, Demaegdt, Daminet et al. 2016, Urbanek, Habrowska-Górczyńska et al. 2018). Desta maneira, estudos adicionais sobre a toxicidade reprodutiva destas micotoxinas são necessários para que estratégias futuras possam ser elaboradas a fim de minimizar os possíveis impactos causados pela exposição a estas toxinas.

Todas as espécies de animais avaliados são susceptíveis aos efeitos tóxicos de DON, na seguinte ordem de susceptibilidade: suínos > camundongos > ratos > aves ≈ ruminantes (Eriksen, Pettersson et al. 2004). Em adição, fatores relacionados ao hospedeiro tais como sexo, idade, estado nutricional e doenças pré-existentes podem influenciar os efeitos *in vivo* de DON. Em animais expostos a DON, suínos e camundongos machos apresentam maior sensibilidade a perda de peso e consumo alimentar do que as fêmeas (Cote, Beasley et al. 1985, Clark, Flannery et al. 2015, Pestka, Clark et al. 2017). Entretanto, trabalhos adicionais sobre os efeitos tóxicos sistêmicos de DON considerando os diferentes sexos não foram localizados na literatura. Ainda vale ressaltar que de acordo com Escrivá, Font et al. (2015), 54% de todos estudos publicados na última década sobre a toxicidade de fusariotoxinas têm utilizado somente machos, 15% usaram apenas fêmeas, 15% usaram uma mistura de ambos os sexos e em 15% destes estudos o sexo não foi especificado. Desse modo, o sexo é um fator que tem sido negligenciado em grande parte nos estudos micotoxicológicos, em que em geral, as fêmeas estão sub-representadas.

Considerando que um indivíduo em fase de crescimento apresenta uma maior necessidade de calorias com base no peso corporal do que um adulto e assim poderá consumir mais micotoxinas, a questão da exposição juvenil a DON requer atenção. Especificamente, diferenças relacionadas à idade têm sido observadas sobre a absorção gastrointestinal, metabolismo e excreção renal de xenobióticos (Strolin, Whomsley et al. 2005). Reforçando este fato, a exposição de camundongos desmamados a 5 mg DON/kg de peso corporal via oral induziu um aumento de 2 a 3 vezes na expressão de citocinas esplênicas (TNF-α, IL-1b e IL-6) comparado aos animais adultos (Pestka ,Amuzie 2008).

Ainda é importante destacar que de acordo com estudos recentes em países como Brasil, Noruega e Reino Unido, crianças e adolescentes comparados a adultos e idosos apresentam um risco aumentado de exposição a DON (Sundheim, Lillegaard et al. 2017, Papageorgiou, Wells et al. 2018, Silva, Pante et al. 2018). De acordo com Silva, Pante et al. (2018), no estado do Paraná o consumo de DON por indivíduos com 10 a 19 anos do sexo masculino e feminino excede o TDI (ingestão diária tolerável) estabelecido para DON (JECFA, 2011). Em parte, este resultado, pode estar relacionado ao fato que o estudo utilizado para o cálculo do TDI de DON (1 µg de DON/kg de peso corpóreo por dia) (Joint, Additives et al. 2011) tenha sido baseado somente sobre efeitos gerais e imunotóxicos observados em camundongos alimentados por uma dieta contaminada por dois anos, em que não foi levado em consideração animais de diferentes faixas etárias. Assim, relacionar as diferenças entre os sexos ratos durante o período juvenil e peripuberal com o aumento da vulnerabilidade a DON é uma consideração importante para a elaboração de regulamentos que visem controlar a exposição humana a esta micotoxina.

Em nível celular, DON liga-se a subunidade ribossomal 60S, o que pode inibir a síntese de proteínas, induzindo fosforilação das proteínas quinases ativadas por mitógenos (MAPKs) em um processo conhecido como estresse ribotóxico (Pestka 2007). Por meio da ativação de MAPKs, DON ainda pode regular a expressão de citocinas inflamatórias e quimiocinas, promovendo apoptose (Zhou, Islam et al. 2005). Em adição, estudos prévios na última década têm reportado que o estresse oxidativo (EO) é também um importante mecanismo subjacente na toxicidade de DON (Mishra, Dwivedi et al. 2014). DON é capaz de gerar radicais livres, incluindo espécies reativas de oxigênio (ROS) em vários tipos celulares, o que induz a peroxidação lipídica levando a alterações na integridade de membrana e no status antioxidante das células (Krishnaswamy, Devaraj et al. 2010, Li, Ma et al. 2014). Desse modo, elucidar o envolvimento do EO induzido por DON em modelo *in vivo* é de extrema importância para a compreensão dos mecanismos toxicológicos envolvidos na toxicidade desta micotoxina em animais e humanos.

1.1 Toxicidade de DON sobre ovários de mamíferos

Durante a década de 80, trabalhos *in vivo* sobre a toxidade reprodutiva de DON foram conduzidos por Morrissey ,Vesonder (1985) e Friend, Trenholm et al. (1986). De acordo com Morrissey ,Vesonder (1985), em machos e fêmeas de ratos Sprague Dawley expostos uma dieta contaminada (5 mg de DON/kg de ração), DON foi responsável em prejudicar o índice gestacional, uma vez que somente 50% dos acasalamentos dos animais contaminados resultaram em gestação, comparado com os 80% no grupo controle. Em contrapartida, quando se avaliou a morfologia de ovários e testículos de suínos expostos a dietas contendo

3,7 e 4,2 mg de DON/ kg de ração por 7 semanas, respectivamente, nenhuma alteração morfológica nas gônadas feminina e masculina foi observada por Friend, Trenholm et al. (1986).

No início do século XXI estudos sobre a toxicidade reprodutiva de DON começaram a ser desenvolvidos por meio de modelos *in vitro*. Em estudo conduzido por Alm, Greising et al. (2002), DON foi associado a um efeito inibitório potente sobre a maturação de oócitos suínos, significativamente diminuindo a proporção de oócitos na metáfase II. Em um estudo subsequente, para avaliar a toxicidade de DON durante estágios específicos do desenvolvimento folicular, complexos cumulus oócitos coletados de ovários de suínos foram expostos a 0,02-0,2 e 2 µM DON (Schoevers, Fink-Gremmels et al. 2010). Na maior concentração, DON inibiu a expansão do cumulus induzindo morte celular (Schoevers, Fink-Gremmels et al. 2010). Em adição, DON em todas as concentrações resultou em menos oócitos de metáfase II, concordante com os resultados obtidos por Alm, Greising et al. (2002) e levou a malformações do fuso mitótico como previamente observado por Malekinejad, Schoevers et al. (2007). Quando oócitos foram expostos a 2 µM de DON, aberrações no fuso ocorreram na metáfase I, e oócitos na presença de 0,2 ou 0,02 µM de DON exibiram aberrações no fuso após alcançarem a metáfase II (Schoevers, Fink-Gremmels et al. 2010).

Além disso, DON tem mostrado ter um efeito dose dependente sobre a proliferação de células granulosas de suínos, esteroidogênese e expressão gênica (Ranzenigo, Caloni et al. 2008, Medvedova, Kolesarova et al. 2011). Em estudo conduzido por Ranzenigo, Caloni et al. (2008), DON teve um efeito bifásico sobre o crescimento celular com concentrações de 0,034 e 0,34 µM aumentando o número de células e 3,4 µM drasticamente diminuindo o número de células. DON em 0,34 e 3,4 µM inibiu a produção de progesterona e estradiol induzida por FSH e IGF-1 e em 3,4 µM bloqueou completamente a abundância de mRNA CYP19A1 e CYP11A1 induzida por FSH e IGF-1 (Ranzenigo, Caloni et al. 2008). Ao contrário, Medvedova, Kolesarova et al. (2011) observou que DON na concentração de 3,4 µM aumentou a expressão de marcadores de proliferação celular (ciclina B1 e PCNA) e estimulou a liberação de progesterona em células granulosas de suínos. No entanto, isso ocorreu em condições experimentais diferentes do estudo realizado por Ranzenigo, Caloni et al. (2008), onde as células da granulosa foram expostas a DON na presença de soro bovino fetal. Diferentemente das células da granulosa suína cultivadas em meio sem soro que são dependentes da síntese de novo colesterol para a esteroidogênese (Baraño, Hammond, 1986), as células cultivadas em meio contendo soro são dependentes de colesterol de lipoproteína exógena para a biossíntese de esteroides (Veldhuis et al., 1984).

Desse modo, movidos pela necessidade da padronização de outros modelos experimentais no estudo da toxicidade reprodutiva de DON em suínos, nosso grupo de pesquisa avaliou o efeito de DON (10 µM) sobre explantes ovarianos de suínos em que o padrão de diferenciação celular visto *in vivo* é mantido (Gerez, Desto et al. 2017). Então, após 48 horas de exposição, DON induziu uma redução no número de folículos normais e aumento no número de oócitos picnóticos em todos os estágios do desenvolvimento folicular. Resultado similar foi observado por Kolesárová, Capcarová et al. (2012) em que explantes ovarianos de ratos expostos a DON (0,034 - 0.34 e 3.4 µM) apresentaram uma expressão aumentada de proteínas apóptoticas (Bax e caspase-3) e uma redução na expressão de peptídeo antiapoptótico (Bcl-2). Em contraste, oócitos de suínos expostos a DON (3 µM) não apresentaram nenhuma alteração na expressão de caspase-3, mas um aumento na expressão de genes relacionadas a autofagia (Lamp2 e LC3) foi observado (Han, Wang et al. 2016). Adicionalmente, de acordo com Lan, Han et al. (2018) DON induz apoptose em oócitos de camundongos por meio de um aumento nos níveis de ROS. Interessantemente, quando o tratamento com DON foi associado a melatonina, um antioxidante natural, a apoptose mediada pelo estresse oxidativo foi inibida. Assim, pode-se concluir que nos ovários, DON é capaz de induzir morte celular por diferentes vias como apoptose e autofagia sendo o estresse oxidativo um mecanismo importante na toxicidade reprodutiva desta micotoxina.

1.2 Toxicidade de DON sobre testículos de mamíferos

Sprando, Pestka et al. (1999) avaliaram em três linhagens de camundongos machos

(B6129-IL6: deficiente para o gene Il-6; B6129F2: B6129-IL6 com o gene IL-6 intacto; B6C3F1: controle) o efeito de DON sobre a morfologia testicular e sobre a contagem de espermatozoides do testículo e epidídimo. Após 90 dias de exposição a um alimento contaminado com 10mg/kg de DON, o diâmetro dos túbulos seminíferos, a altura do epitélio seminífero e o número de células de Sertoli no grupo DON não diferiram significativamente dos animais controle. No entanto, em ratos machos expostos a DON (2,5 e 5,0 mg/kg) via intubação gástrica por 28 dias, uma redução no peso do epidídimo e vesícula seminal, foi reportado por Sprando, Collins et al. (2005). Nos animais expostos a maior dose (5,0 mg/kg de DON) uma diminuição considerável na contagem espermática testicular, número de espermátides e espermatozoides da cauda do epidídimo foi verificado. Além disso, o numero de espermatozoides com cauda quebrada foi significativamente maior enquanto a velocidade de batimento do flagelo foi consideravelmente menor comparado ao grupo controle (Sprando, Collins et al. 2005). Interessantemente, alterações morfológicas foram acompanhadas por alterações hormonais, pois a concentração do hormônio folículo estimulante e luteinizante estava aumentada enquanto a concentração sérica de testosterona diminuiu em todos os grupos experimentais de maneira dose dependente (Sprando, Collins et al. 2005).

Então, usando uma cultura de células H295R o potencial de DON (0,1 a 1000 ng/ml) em desregular o sistema endócrino foi verificado por (Ndossi, Frizzell et al. 2012). Neste estudo *in vitro*, concentrações aumentadas de DON foram capazes de reduzir progressivamente a produção de estradiol, testosterona e cortisol, além de regular significativamente treze dos dezesseis genes esteroidogênicos. Em seguida, efeitos inibitórios de DON sobre a viabilidade celular e a produção de progesterona em linhagem de células Leydig (MA-10) foram verificados por Savard, Nogues et al. (2016).

Somados aos danos reprodutivos de DON no sistema reprodutor masculino, estudos recentes sobre os seus mecanismos toxicológicos têm sido realizados (Hallaj Salahipour, Hasanzadeh et al. 2019, Yang, Wang et al. 2019, Cao, Huang et al. 2020). Em cultura de espermatozoides de camundongos expostos a DON (2,5; 5; 10 μM) redução na atividade metabólica espermática e aumento na peroxidação lipídica e dano ao DNA foram responsáveis por um declínio dos parâmetros de qualidade espermática. Resultado similar foi verificado por Yang et al. (2019), em que reduzida qualidade espermática e alterações morfológicas testiculares foram acompanhadas por estresse oxidativo testicular, e um aumento nos níveis de JNK/c-Jun e na expressão de genes e proteínas apoptóticas nos testículos de camundongos tratados com DON (via intragástrica na dose de 2.4 mg/kg de peso corpóreo por 4 semanas). Adicionalmente, em um estudo conduzido por Cao, Huang et al. (2020), em que camundongos machos foram expostos intragastricamente a DON (0, 1.2, 2.4 and 4.8 mg/kg peso corpóreo), desordem na espermatogênese foi associada a uma alteração da barreira hematotesticular com diminuição dos níveis de testosterona e inflamação.

1.3 Desoxinivalenol e estresse oxidativo

Após a ingestão de alimento contaminado, células epiteliais do sistema digestório correspondem a primeira barreira e podem ser expostas a altas doses de micotoxinas (Bouhet ,Oswald 2005). Dessa maneira, através de estudos *in vitro* e *in vivo* a participação do estresse oxidativo na toxicidade intestinal de DON tem sido avaliada. Em cultura de células de cólon humano cancerígenas (Caco-2) expostas por 24 h a DON (5-40µM), uma diminuição da viabilidade celular e inibição da síntese proteica e de DNA foi acompanhada por peroxidação lipídica caracterizada por um aumento da produção de malondialdeído (MDA) (Kouadio, Mobio et al. 2005). Em adição, apoptose associada a níveis aumentados de espécies reativas de oxigênio intracelular (ROS) (Tang, Li et al. 2015), MDA e uma diminuição significativa no potencial antioxidante foram verificados em linhagens de células epiteliais intestinais de suíno IPEC-J2 tratadas com DON (0,5 e 1µg/mL) por 6h, 12 e 24 h (Xu, Yan et al. 2020). Resultado similar foi observado por (Wan, Turner et al. 2019), em que o tratamento com DON (1 µM) por 24 h resultou em células de adenocarcinoma coloretal humano HT-29, em uma diminuição da viabilidade celular, níveis aumentados de ROS, óxido nítrico e MDA com alterações significativas nas enzimas antioxidantes.

Assim, movidos pelo interesse em mimetizar o consumo de um alimento contaminado por animais e humanos, estudos subsequentes têm avaliado o efeito oxidativo de DON em explantes intestinais de suínos (da Silva, Gerez et al. 2019) e células HIEC-6 (origem de intestino fetal humano) (Pomothy, Szabó et al. 2021). Em explantes intestinais de suínos o tratamento com DON (10µM) por 4h induziu estresse oxidativo o que foi responsável por um aumento de células apoptóticas e alterações histológicas significativa (da Silva, Gerez et al. 2019). Em linhagem celular HIEC-6 foi demonstrado que após 24h de tratamento com 1 µM DON, esta micotoxina foi responsável em causar estresse oxidativo caracterizado por um aumento dos níveis de ROS intracelular e da concentração extracelular de peróxido de hidrogênio (Pomothy, Szabó et al. 2021). No entanto, até o presente momento na base de dados consultadas, estes resultados *in vitro* foram verificados em modelo *in vivo* usando animais monogástricos somente em um único estudo. De acordo com Wu, Xiao et al. (2014) em leitões submetidos a uma dieta contaminada com 4 mg de DON/kg por 37 dias, lesões histopatológicas intestinais foram acompanhadas por níveis aumentados de MDA e capacidade antioxidante reduzida.

O papel do estresse oxidativo tem sido avaliado no dano hepático induzido por DON. Em cultura de hepatócitos de ratos clone-9 expostos a esta toxina (0–100 µg/ml) por 48 h o efeito genotóxico foi relacionado com um aumento do estresse oxidativo dependente da concentração (Sahu, Garthoff et al. 2008). Resultado similar foi observado em células HepG2 (linhagem de hepatocarcionama humano), em que o estresse oxidativo foi confirmado e a genotoxidade causada por DON (3,75–30 µM) prevenida significativamente com pré-tratamento contendo o antioxidante hidroxititosol (Zhang, Jiang et al. 2009). Em contraste, quando diferentes linhagens de hepatócitos oriundas de rato (Clone9 e MH1C1), camundongo (NBL e CL2) e humano (WRL68 e HepG2) foram tratadas por 24 h (0–25 µg/ml), o estresse oxidativo não mostrou participação na hepatotoxidade de DON (Sahu, O'Donnell et al. 2010). Ratos tratados oralmente por 3 semanas com 5 mg de DON/Kg de peso corpóreo, o estresse oxidativo foi confirmado em modelo *in vivo* como o mecanismo subjacente na hepatotoxidade de DON (Abdel-Wahhab, El-Nekeety et al. 2018). Estes dados foram confirmados em um recente estudo, em que a lesão no fígado de ratos expostos via oral a 2,4 mg DON/kg de peso corpóreo por 28 dias foi causada via estresse oxidativo, que por sua vez foi caracterizado por uma diminuição das enzimas antioxidantes e ativação da via de sinalização Keap1-Nrf2 (fatores transcrição envolvidos na regulação do equilíbrio redox celular) (Bai, Ma et al. 2021). Em contraste, nenhum efeito foi observado na atividade antioxidante total no fígado de leitões (dieta contendo 4 mg/kg por 37 dias) embora um aumento nos níveis de MDA e H_2O_2 tenha sido observado no tecido hepático (Wu, Xiao et al. 2014).

Considerando ainda que DON pode ser metabolizado além do fígado nos rins (Rotter et al., 1996), estudos prévios têm avaliado a participação do estresse oxidativo na nefrotoxicidade de DON. Em modelo *in vivo* apoptose, disfunção renal e estresse oxidative foi observado nos rins de camundongos expostos a uma intoxicação aguda (Liang, Ren et al. 2015). Em adição, estresse oxidative tem sido associado com a nefrotoxicidade de DON em suínos alimentados com uma dieta contaminada contendo 4 mg DON/kg de alimento por 37 dias (Wu et al. 2014).

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar a toxicidade reprodutiva e sistêmica de fusariotoxinas sobre explantes ovarianos (DON e FB_1) e em ratos machos e fêmeas durante o período juvenil e puberal (DON).

2.2 Objetivos Específicos

- Avaliar o efeito de DON e ou FB_1 na integridade folicular, proliferação celular e apoptose de explantes ovarianos de suínos de terminação;
- Avaliar o efeito de DON e ou FB_1 na resposta oxidativa e antioxidante de explantes ovarianos de suínos de terminação;
- Avaliar a integridade folicular e mecanismos apoptóticos em ovários de ratas alimentadas com uma dieta contaminada com DON durante o período juvenil e peripuberal;
- Avaliar a integridade testicular e apoptose nos testículos de ratos alimentados com

uma dieta contaminada com DON durante o período juvenil e peripuberal;

- Avaliar o efeito de DON sobre parâmetros bioquímicos, hematológicos e histológicos de ratos machos e fêmeas alimentados com uma dieta contaminada com DON durante o período juvenil e peripuberal;
- Avaliar o efeito de DON sobre estresse oxidativo intestinal, hepática e renal de DON de ratos alimentados com uma dieta contaminada com DON durante o período juvenil e peripuberal.

3. MATERIAL E MÉTODOS

3.1 Reagentes

DON (peso molecular 296,32; Sigma Aldrich St. Louis, MO, USA) e FB_1 purificados (peso molecular 721,83; Cayman chemical company, Ann arbor, MI, USA) foram dissovidos em água ultrapura na concentração de 10 µM para DON e 100 µM para FB_1 e estocadas a 4ºC.

3.2 Animais e delineamento experimental

Neste projeto foram realizados 2 experimentos independentes:

Experimento 1 (*ex vivo*): Ovários (n = 6 pares de ovários) foram coletados em abatedouro local de seis suínos (Landrace x Large White x Duroc) em boas condições corporais, desde que apresentassem corpo lúteo. Então os ovários coletados foram lavados em álcool 70% seguido de 2 lavagens com meio essencial mínimo de cultura (MEM; Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/l, pH 7.2) suplementado com 200 mg/ml penicilina and 200 mg/ml streptomicina. Os ovários então foram transportados em MEM a 20ºC, dentro de 1 hora ao laboratório. No laboratório, cada ovário foi cortado longitudinalmente, e após a remoção de folículos antrais grandes, corpo lúteo e medula, o córtex ovariano foi dividido em fragmentos de aproximadamente 3 x 3 x 1 mm. Em seguida, os explantes foram incubados em placas de seis poços (dois explantes/poço) com 5 ml de meio de cultura MEM, suplementado com ITS (insulina 6,25 mg/mL, transferrina 6,25 mg/mL e selênio (6,25 ng/mL), 0,23 mM piruvato, 2 mM glutamina, 2 mM hipoxantina, 1,25 mg/mL albubina sérica bovina (BSA Gibco BRL, Rockville, MD, USA), 20 IU/mL penicilina e 200 mg/mL streptomicina. Todos os explantes foram incubados por um período de 48 horas em estufa a 37°C. Explantes de suínos foram alocados em quatro grupos experimentais: 1) MEM (Controle, n=36); 2) 10 μM de DON (DON, n=36); 100 μM FB_1 (FB_1, n=36); 10 μM de DON+ 100 μM FB_1 (DON+FB_1). Um total de 18 explantes por tratamento foram analisados (12 amostras para a histopatologia e 6 para análise do estresse oxidativo.

Experimento 2 (*in vivo)*: 12 ratos fêmeas e 12 ratos machos Wistar, com 21 dias de idade, foram obtidos do Biotério Central – UEL e mantidos em condições adequadas por 7 dias antes do início do período experimental. Os animais foram alimentados com uma dieta padrão (Quimtia®) e água *ad libitum*. Durante o experimento, os animais foram mantidos em temperatura adequada ((± 21°C), ventilação adequada (exaustor de ar), umidade (40-70%) e fotoperíodo 12 h de claro e 12 h de escuro. O projeto foi previamente e aprovado pelo comissão de ética de uso animal da nossa instituição (CEUA protocol nº 6986.2017.27). Então, após os 7 dias de adaptação os animais com 28 dias de idade foram randomizados em 2 grupos: Grupo controle (n = 12, 6 fêmeas e 6 machos, dieta livre de micotoxina e Grupo DON: (n = 12, 6 fêmeas e 6 machos, dieta contendo 10 mg DON/kg de alimento. Um extrato cru de DON (40 ppm) produzido no Laboratório de Micologia, Colégio de Agricultura Luiz de Queiroz, Universidade São Paulo, foi usado na preparação da dieta contaminada. Uma mistura (dieta padrão+DON) foi preparada na Universidade Estadual de Londrina usando um mixer de ração. As amostras da dietas foram enviadas para o Laboratório Samitec (Santa Maria – RS/Brasil), onde os níveis de micotoxinas foram determinados por HPLC/método MS-MS). A dieta controle mostrou níveis mais baixos de DON do que o limite de quantificação estabelecido (200 μg/kg com um estado de acurácia de 101%). Após 28 dias de experimento, os animais foram eutanasiados usando overdose com pentobarbital sódico, o que foi confirmado por deslocamento do segmento cervical e decapitação. Então, amostras de sangue, intestino, fígado, rim, ovário e testículos foram colhidos e destinados para análises posteriores. No entanto, no momento da colheita ovários e testículos foram pesados em balança analítica.

3.3 Análise morfológica e morfométrica

Os explantes ovarianos intestino, fígado, rim, ovários e testículos foram fixados em solução de formalina tamponado 10% em torno de 24 h, e após a fixação, os tecidos foram destinados a rotina histológica padrão e confecção de lâminas histológicas coradas em hematoxilina e eosina. Alterações histológicas de explantes ovarianos foram avaliadas usando um escore lesional de acordo com (Gerez, Desto et al. 2017). Estas alterações foram também avaliadas baseadas no estágio de desenvolvimento follicular. Então os folículos ovarianos foram classificado de acordo com (Phoophitphong, Wangnaitham et al. 2012) em: primordial, primário, em crescimento (pre-antral e antral). A integridade folicular foi avaliada em relação a integridade da membrana basal, presença ou ausência de núcleos picnóticos e integridade do oócito e das células da camada granulosa (Gerez, Pinton et al. 2015). Assim, cada folículo na lâmina histológica foi classificado como: Normal; degenerado tipo 1 (oócito degenerado com células granulosas aparentemente normais); degenerado tipo 2 (oócito e células granulosas degeneradas; degenerado tipo 3 (oócito aparentemente normal e células granulosas degeneradas). Nos ovários dos animais os folículos foram divididos em cinco categorias de acordo com a classificação de Pedersen ,Peters (1968): (1) folículo primordial e primário (2) folículos em crescimento; (3) folículo pré-antral; (4) folículo antral; (5) folículo maduro. Em adição, cada folículo foi classificado como normal e atrésico. Então, em cada lâmina, o número total de cada tipo folicular e corpo lúteo foi observado e após a análise de 10 cortes de cada animal obtidos no intervalo de 10 cortes, uma média de cada tipo folicular e corpo lúteo foi obtido por animal.

Nos testículos a dinâmica da espermatogênese foi realizada em 100 secções tubulares aleatórias por animal que foram classificadas em quatro categorias: estágios I-VI, VII-VIII, IX-XIII e XIV do ciclo do epitélio seminífero (Leblond ,Clermont 1952). Para análise morfométrica dos testículos avaliou-se a altura do epitélio germinativo e o diâmetro dos túbulos seminíferos. A análise foi realizada em túbulos em fase IX de cada testículo. Em cada túbulo seminífero obteve-se quatro medidas de altura e quatro medidas de diâmetro para a realização de média e análise estatística (Favareto, Fernandez et al. 2011). As análises foram realizadas com a utilização de um microscópio óptico Leica com sistema de imagem MOTIC plus (Richmond, Canada). O número de células de Leydig foi contabilizado em 10 campos aleatórios de tecido intersticial por animal utilizando-se a objetiva de 40x e um microscópio óptico Leica com sistema de imagem MOTIC plus (Richmond, Canada) (Guerra, Sanabria et al. 2017). A contagem do número de células de Sertoli foi determinada em 20 cortes transversais de túbulos seminíferos na fase VII-VIII para cada testículo com o auxílio também da objetiva 40x (Nassr, Arena et al. 2010).

Para avaliação do intestino (jejuno), fígado (parte medial do lobo esquerdo) e rins (esquerdo) três lâminas coradas em hematoxilina e eosina por animal foram analisadas. Para a análise de cada órgão, um escore morfológico foi usado (Grenier, Loureiro-Bracarense et al. 2011, Gerez, Pinton et al. 2015), onde a frequência e severidade de cada critério foi considerado por um escore predeterminado: 0 (ausente), 1 (discreto), 2 (moderado), and 3 (severo).

3.4 Análise histoquímica

Com objetivo de avaliar a proliferação celular em explantes ovarianos e testículos, lâminas histológicas foram coradas com nitrato de prata como descrito previamente por Crocker ,Skilbeck (1987) e as regiões organizadoras de nucléolo foram identificadas ao microscópio óptico. O número de NORs foi contado em 100 células granulosas de todos os folículos em crescimento na amostra no aumento de 1000x. Os resultados foram expressos como o número de NORs por célula em diferentes tratamentos. Já nos testículos dos animais, a quantificação das regiões organizadoras de nucléolos (NORs) foi realizada aleatoriamente em cinco túbulos de cada estágio da dinâmica da espermatogênese (exceto o estágio XIV devido à impossibilidade de localizar a meiose II dos espermatócitos II, característica desse estágio). Em cada túbulo quantificou-se o número de NORs em 20 células, totalizando 100 células por estágio.

3.5 Análise imunohistoquímica

Nos explantes ovarianos e testículos células apoptóticas foram avaliadas usando um anticorpo anti-caspase 3 clivado (CCasp3, clone Asp 175, Cell Signaling Technology, Beverly, MA) de acordo com (da Silva, Gerez et al. 2014). Imunomarcação citoplasmática nas células de folículos primordias, primário e em crescimento de explantes ovarianos foi usado para avaliar o index celular apoptótico em 10 campos aleatórios usando um aumento de 1000x. Somente células granulosas mostrando marcação forte e homogênea foram consideradas positivas. Nos túbulos seminíferos a análise de imunomarcação para caspase-3 foi realizada em 300 túbulos seminíferos que foram classificados em imunomarcados ou não. Os túbulos com imunomarcação foram posteriormente submetidos a mesma classificação utilizada na dinâmica da espermatogênese (Dutra Gonçalves, Antunes Vieira et al. 2017). Nos ovários dos animais biomarcadores de morte celular como BAX, caspase-3 (indutores de apoptose) e BCL-2 (inibidor de apoptose) foram analisados de acordo com o protocolo de (Chuffa, Alves et al. 2016). Neste órgão, todos as células foliculares e luteínicas presentes nos folículos ovarianos e corpo lúteo foram contados. Usando o mesmo critério, a contagem de células positivas para BCL-2, BAX, and caspase-3 foi realizada. Através do número de células positivas para BCL-2, BAX, and Caspase-3 pelo número total de células de cada folículo ovariano ou corpo lúteo, a porcentagem média (%) de imunomarcação para cada proteina presente nos folículos ovarianos e corpo lúteo foi calculado. Olhando as células estromais e oócitos imunomarcados, a análise foi realizada baseada na intensidade de coloração que foi classificada como ausente, fraca, moderada e forte. Sempre que possível, 10 campos foram analisados em cada corte histológico.

3.6 Parâmetros zootécnicos

Durante o experimento *in vivo*, 2 animais do mesmo sexo foram mantidos na mesma caixa e para a análise zootécnica, a caixa foi considerada o experimento. Os animais foram pesados semanalmente e o ganho de peso corpóreo e consumo alimentar foi calculado por semana.

3.7 Hematologia e bioquímico

A contagem de células sanguíneas (CBC) foi realizada imediantamente após a colheita sanguínea (punção cardíaca) em um aparelho automatizado poCH-100*iV Diff*™ (Sysmex, Kobe, Japan) para a contagem de células sanguíneas vermelhas (RBC) e células sanguíneas brancas (WBC), hematócrito (HCT), hemoglobina (HGB), volume corpuscular médio (MCV), corpuscular hemoglobina corpuscular média (MCH) e a concentração de hemoglobina corpuscular media (MCHC). Contagem diferencial de WBC e estimação de plaquetas (PLT) foram manualmente realizadas em esfregaços sanguíneos corados com Diff-Quick (NEWPROV, Paraná, Brazil). Os testes bioquímicos foram realizados em aparelho de análise química Dimension® Xpand (SIEMENS, Siemens Healthcare Diagnostics Inc., PA, USA) para determinar as seguintes análises plasmáticas: creatinina, ureia (BUN), ácido úrico, glicose, fosfatase alcalina (ALP), alanine aminotransferase (ALT), aspartato aminotransferase (AST), colestrerol, lipoproteina de alta densidade (HDL) e triglicerideos.

3.8 Análise do estresse oxidativo

Explantes ovarianos e amostras (n=4/grupo) de jejuno, fígado (parte medial do lobo esquerdo) e rim (direito) foram colhidos em microtubos estéreis, mantidos em -80ºC. Para a capacidade antioxidante, os testes de glutationa reduzida (GSH) de acordo com (Sedlak ,Lindsay 1968), capacidade de redução férrica (FRAP) e redução of 2,2′-azino-bis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS) seguindo as metodologias descritas por Katalinic, Modun et al. (2005) foram realizadas. Nas amostras de jejuno, fígado e rim a produção do ânion súperóxido foi determinada pelo teste *nitroazul* de *tetrazólio* (NBT) (Fattori et al., 2015). Peroxidação lipídica foi avaliada pelo nível de substância reativas ao ácido tiobarbitúrico (TBARS) (Manchope, Artero et al. 2018). Em resumo, usando um triturador de tecido (Bjospec, São Paulo, SP, Brazil) as amostras foram homogeinizadas em tampão KCL (1.15%) para FRAP, ABTS, NBT e TBARS, e tampão EDTA (0.02 M) para o teste GSH teste, seguindo o protocolo descrito pelos autores citados acima.

3.9 Análise estatística

A homogeneidade de variância e normalidade de resíduos foram inicialmente analisados através dos testes de Bartlett e de Shapiro, respectivamente. Para dados paramétricos teste T de student (comparação de 2 grupos experimentais) ou teste de Tukey (comparação de 3 ou mais grupos experimentais) foi usado. Para dados não-paramétricos, teste de Kruskal-Wallis e Dunn foram aplicados. Em adição, quando o efeito de variáveis independentes sobre uma váriavel dependente foi investigado análise fatorial foi aplicada. Para todos os testes nível de significância de 0,05 foi considerado.

4. RESULTADOS E DISCUSSÃO

Os resultados obtidos no doutoramento em Ciências da Saúde estão descritos em quatro artigos científicos.

4.1. Ovarian toxicity by fusariotoxins in pigs: does it imply in oxidative stress? Juliana Rubira Gerez, Thaynara Camacho, Victor Hugo Brunaldi Marutani, Ricardo Luís Nascimento de Matos, Miriam Sayuri Hohmann, Waldiceu Aparecido Verri Jr., and Ana Paula F. R. L. Bracarense. Artigo publicado em 16/02/2021 no periódico Theriogenology (fator de impacto: 2,74 e Qualis-periódicos B2 em medicina. https://doi.org/10.1016/j.theriogenology.2021.02.003

4.2 Effects of deoxynivalenol exposure at peripuberty over testicles of rats: structural and functional alterations. J.R. Gerez, A.L.P.L Gomes, R.P. Erthal, G.S.A. Fernandes, R.L.N. Matos, W.A. Verri Jr, E.M Gloria., A.P.F.R.L. Bracarense. Artigo publicado em 05/08/2021 no periódico World Mycotoxin Journal (fator de impacto: 3.353 e Qualis-periódicos B1 em medicina II). https://doi.org/10.3920/WMJ2020.2667

4.3 Exposure to deoxynivalenol during peripubertal periods induces ovarian damage in immature rats. Gerez J.R.; Camacho T.; Marutani V.H.; Chuffa L.G.A.; Silveira H.S.; Verri Jr, W.A.; Gloria E.M.; Bracarense A.P.F.R.L. Artigo que será submetido no periódico Reproduction in domestic animals (Fator de impacto 2.005 e Qualis-periódico B2 em medicina I).

4.4 Animal performance and clinical response to deoxynivalenol is sex-dependent in peripubertal rats. Gerez, JR, Verri, W, Hohmann, M.S., Flaiban, K. M. C., Hasuda, A.L., Gloria E.M., Bracarense, A.P.R.L. Artigo que será submetido no periódico Toxicon (Fator de impacto 3.033 e Qualis-periódico B1 em medicina I).

4.1 Ovarian toxicity by fusariotoxins in pigs: does it imply in oxidative stress?

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Abstract

Mycotoxins are natural contaminants of food and feed occurring worldwide. Deoxynivalenol (DON) and fumonisin B1 (FB1) are the most frequent fusariotoxins and induce immune and intestinal toxicity in humans and animals. Recently, an association between mycotoxins exposure and impaired fertility has been suggested. However, the effects of these mycotoxins on the reproductive system are not well established. This study aimed to evaluate the effects of FB1 and DON, in combination or alone, on the ovarian morphology and oxidative responses using porcine explants. Seventy-two explants were obtained from six pigs and submitted to the following treatments: control (MEM medium), DON (10 μM), FB1 (100 μM FB1), and DON+FB1 (10 μM+100 μM). Histological and immunohistochemical assays were performed to evaluate ovarian changes, cell proliferation, and apoptosis. Oxidative stress response was evaluated through lipid peroxidation and antioxidant capacity response assays. The exposure to mycotoxins induced significant histological changes in the ovaries, which were characterized by a decrease in viable follicles and increase in degenerated follicles. A significant decrease in granulosa cell proliferation was observed in explants exposed to all mycotoxins, whereas no change occurred in the cell apoptosis index. The FB1 and multi-contaminated treatments induced a significant decrease in lipid peroxidation accompanied by an increase in antioxidant responses. Altogether, our results indicate a reproductive toxicity induced by fusariotoxins. Moreover, mycotoxins, alone or in combination, modulate oxidative stress response, interfering with the production of free radicals and affecting the reproductive capacity of pigs.

Keywords: mycotoxins, reproductive system, antioxidant potential.

1. Introduction

Infertility is an increasingly serious public health concern affecting around 48.5 million couples worldwide [1]. A recent study focusing on infertility prevalence showedincreasing rates from 1990 to 2017, affecting 0.37% of women and 0.29% of men per year [2]. Numerous synthetic (pesticides, industrial chemicals, bisphenols) and natural (mycotoxins, phytoestrogens) substances have been implicated in endocrine disruptive effects, producing developmental and reproductive changes in humans and animals [3, 4].

Fusarium mycotoxins, which include fumonisin B1 (FB1), zearalenone (ZEA), and trichothecenes such as deoxynivalenol (DON) produced mainly by molds of the *Fusarium* genus, are contaminants in food and feed commodities throughout the world [5]. Upon the consumption of contaminated grains or food products, *Fusarium* mycotoxins can cause a variety of adverse effects and pose serious health hazards to both humans and livestock [6]. Although *Fusarium* mycotoxins have been considered as a potential regulator of intracellular steroidogenesis, studies onreproductive toxicity caused by DON and FB1 in animals are scarce and mainly based on *in vitro* models [4, 7-9]. This way, additional studies on reproductive toxicity associatedwith these mycotoxins are needed to identify adverse reproductive health effects and avoid further harmful chemical exposures.

DON is one of the most frequent contaminants of grains, decreases protein synthesis, and induces a ribotoxic stress response [10]. It also reduces reproductive performance in mammals by impairing estrus cycle, oocyte maturation, and embryo development [11-15]. In *in vitro* studies, direct effects of DON on secretions of growth factor IGF-I and progesterone, as well as on cell proliferation and apoptosis, have been observed in porcine oocytes and granulosa cells [16, 17]. Furthermore, studies using several cell lines have reported that oxidative stress is also an important mechanism underlying DON-induced cytotoxicity and apoptosis [18, 19]. However, surprisingly no report has been released about the participation of oxidative stress in reproductive toxic effects of DON on swine ovarian tissue.

FB1 is a common contaminant of maize and maize-products found in co- occurrence with other *Fusarium* toxins, such as DON [20]. FB1 presents strong similarityto sphinganine and sphingosine, inhibiting the ceramide synthase pathway, resulting in an accumulation of sphingoid bases, which are pro-apoptotic, cytotoxic growth inhibitors,and immunotoxic [21]. Additionally, the potential of FB1 to induce oxidative stress accompanied by ROS generation, cytotoxic effects, and apoptosis has been supported by *in vitro* and *in vivo* studies [22]. Although FB1 induces inhibitory effects on porcine granulosa cell proliferation and delay attainment of sexual maturity in growing pigs [23,24], its mechanism of action on ovarian tissue has not been studied so far.

The effects of *Fusarium* mycotoxins on animal ovaries are poorly understood. Afew studies have focused on the combined effects of fusariotoxins on the female reproductive system [25]. Considering that 10 to 30% of infertile couples present unexplained infertility, which has been significantly associated with human exposure to different environmental toxins [26-28], more studies on the reproductive effects of mycotoxins are needed. Therefore, the purpose of this study was to determine the effects of individual and combined exposure of DON and FB1 on the morphology, proliferation, and apoptosis of ovarian explants of pigs. It also aimed to provide the first information about the individual and combined effects of DON and FB1 on the oxidative stress response in pig ovarian tissue.

2. Materials and methods

2.1 Ovarian samples and reagents

Ovaries (n= six pairs) were collected at a local slaughterhouse from six 5-month-old nonpregnant sows (Landrace x Large White x Duroc). Immediately after slaughter, the collected ovaries were washed in 70% ethanol followed by two washes with phosphate-buffered saline (pH=7.2). Then, these ovaries were transported in minimal essential medium (MEM; Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/L, pH 7, supplemented with 200 mg/mL penicillin and 200 mg/mL streptomycin) at 20°C, within 1 hour, to the laboratory. The purified DON (Molecular weight (MW): 296.32; Sigma–Aldrich, St. Louis, MO, USA) and FB1 (MW: 721.83; Cayman Chemical Company, Ann Arbor, MI, USA) were dissolved in ultrapure water to final concentrations of 10 µM for DON and 100 µM for FB1 and stored at 4 ºC. The concentrations of DON (10 µM) and FB1 (100 µM) used are equivalent to 3 and 72.18 mg/kg of feed, respectively. These concentrations are in line with our previous studies, in which intestinal, systemic, and reproductive toxicity of fusariotoxins were evaluated using *ex vivo* and *in vivo* models [29-32]. Such concentrations can also be observed in mono or multi-contaminated grains[33, 34].

2.2 Experimental protocol

In the laboratory, each pair of ovaries was sectioned in half, and then each half was divided into fragments of about 3 x 3 x 1 mm, totaling 12 explants per animal. The explants were incubated in six-well plates (two explants/well) filled with 5 mL minimumessential medium, which was control culture medium (MEM, Gibco BRL, Rockville, MD), USA; osmolarity 300 mOsm/L, pH 7.2) supplemented with ITS (insulin 6.25 mg/mL, transferrin 6.25 mg/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 IU/mL penicillin, and 200 mg/mL streptomycin. Subsequently, the obtained samples were divided into the following treatments: control (DMEM medium), FB1 (100 µM), DON (10 µM), and FB1 + DON (100 µM + 10 µM). The explants were incubated for 48 hours in an incubator at 37 °C [30]. This incubation timewas determined through preliminary experiments (data not shown), and is compliant withprevious studies wherein follicular growth was observed in ovine and cattle ovarian tissues incubated for 48 hours [35, 36]. A total of 18 explants per treatment were analyzed(12 samples for histological and 6 for oxidative stress analyses).

2.3 Morphological, histochemical, and immunohistochemical analyses of ovarianexplants

After the incubation period, the explants were fixed in 10% buffered formalin solution for about 24 hours, and after fixation, the tissues were dehydrated in a graded series of alcohol, cleared and diaphanized by xylol, and embedded in paraffin forhistological sections. Afterward, 5-µm sections were stained with hematoxylin and eosinfor histological analysis. Histological changes were evaluated using a lesion score scale,considering the injury intensity as described by Gerez et al. [30]. These changes were alsoevaluated based on follicular development stages. According to the study ofPhoophitphong et al. [37], follicles can be divided into three categories: (1) primordial follicles, which are characterized by oocytes surrounded by a layer of flattened granulosacells on the follicle periphery; (2) primary follicles, which are characterized by a single layer of cuboid granulosa cells around the oocyte; (3) growing follicles, which include preantral follicles (secondary follicles with multiple layers of granulosa cells and an antrum beginning to emerge) and antral follicles (tertiary follicles with multiple layers of granulosa cells and presence of a single large antrum).

Follicular integrity was assessed by verifying the integrity of the basementmembrane, presence or absence of pyknotic nucleus, and integrity of oocyte and granularlayer cells, as described previously [30]. In short, each follicle in the histological sectionwas classified as: (1) normal (healthy oocyte with a non-pyknotic nucleus surrounded bylayered granulosa cells); (2) type I degeneration (degenerated oocyte with apparently healthy granular cells); (3) type II degeneration (degenerated oocyte and granular cells);or (4) type III degeneration (apparently healthy oocyte with degenerated granular cells).

Nucleolar organizing regions (NORs) were stained with colloidal silver nitrate toassess cell proliferation as described previously [38]. The number of NORs was countedin 100 granulosa cells of all growing follicles in the sample at 1000x magnification. Theresults were expressed as the mean number of NORs per cell in different treatments.

Cell apoptosis was evaluated using an antibody anti-cleaved caspase-3 (CCasp3, clone Asp 175, Cell Signaling Technology, Beverly, MA). Briefly, heat-mediated retrieval was performed by heating the sections immersed in citrate buffer (pH 6) in a water bath for 20 minutes. Following, the endogenous peroxidase activity was blocked by incubation in methanol–H_2O_2 solution (11:4). Then, the sections were incubated withprimary antibody (1:200, overnight at 4 ºC). Incubation with polymer secondary antibody(30 min) (Nichirei Biosciences, Tokyo, Japan) was followed by addition of chromogen (3, 3-diaminobenzidine, Invitrogen, São Paulo, SP, Brazil). Finally, the sections were counterstained with hematoxylin. Negative and positive controls were used according to the manufacturer. Cytoplasm immunostaining in the cells of primordial, primary, and growing follicles was used to evaluate cellular apoptotic index in ten random fields at 1000x magnification. Only granulosa cells showing strong and homogeneous staining were considered positive.

2.4 Reduced glutathione (GSH) assay

The GSH levels were evaluated in the ovarian explants using an adapted method previously described by Sedlak and Lindsay [39]. Briefly, with the use of a tissue tearor (Bjospec, São Paulo, SP, Brazil), samples were homogenized in cold EDTA buffer (0.02 M). Next, trichloroacetic acid (50% *w/v*) was added, and then samples were centrifuged (1500 x g for 15 min). The supernatant was mixed with a Tris-HCl solution (0.4 M, pH 8.9) and dithiobisnitrobenzoic acid (10mM). The samples were incubated for 5 min, and their absorbance was read in a spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) at 412 nm. A standard GSH curve was prepared, and the results were presented as nmol of GSH per mg of protein. The intra- and inter-assay coefficients of variation are 3.356% and 9.415%, respectively.

2.5 ABTS and FRAP assays

Antioxidant potential of the samples was evaluated by ferric reducing antioxidantpower (FRAP) and free radical scavenging capacity (ABTS) assays, according to [40]. Initially, the samples were homogenized in 500 µL ice-cold KCl buffer solution (1.15%).For the ABTS assay, 15-µL supernatant was mixed to 200-µL solution containing the reagent 2,2-azino-bis-(3-ethylbenzothiazoline- 6-sulphonic acid) (ABTS) (Sigma Chemical Co., St. Louis, MO, USA), and after 6 min incubation at 25ºC, absorbance wasmeasured at 730 nm. Additionally, 15 µL of the same supernatant was used for FRAP assay. After adding 200-µL FRAP reagent (Sigma Chemical Co., St. Louis, MO, USA),the samples were incubated at 37°C for 30 min, and their absorbance was measured at 595 nm. The results of ABTS and FRAP assays were equated against a standard Troloxcurve (0.02–20 nmol). As these methods are Trolox-equivalent antioxidant capacity (TEAC) assays, the results were presented as nmol Trolox-equivalent/mg protein. The intra- and inter-assay coefficients of variation are 5.373% and 7.493%, respectively, for ABTS assay and 7.575% and 5.914%, respectively, for FRAP assay.

2.6 Lipid peroxidation assay (TBARS)

The levels of thiobarbituric acid-reactive substances (TBARS) in the ovarian explants were evaluated by an adapted method described previously [41]. Initially, aiming to precipitate proteins, trichloroacetic acid (10%) was added to the homogenate, and then the samples were centrifuged (1000 x *g*, 3min, 4 °C). Following sample separation, thiobarbituric acid (0.67%) was added to protein-free supernatant. After 15 min incubation in a water bath at 100 °C, the malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, were determined by difference betweenabsorbances at 535 and 572, using a microplate reader. The results were presented as TBARS (nmolMDA/mg protein). The intra- and inter-assay coefficients of variation are 8.722% and 11.018%, respectively.

2.7 Statistical analysis

The experiment was developed in a completely randomized design, wherein one independent variable (treatment) with four levels (control, DON, FB1, and DON+FB1) was manipulated by researchers. After checking the assumptions of residual normality (Shapiro's test) and homogeneity of variance (Levene's test), the data were represented as mean ± standard error of the mean and analyzed by ANOVA, followed by the Tukey's test for comparison between treatments ($P<0.05$). The lack of normality and/ or homogeneity of the data (number of normal and degenerated follicles according to the stages of follicular development, and number of ovarian follicles and NORs/cell) led to the use of the non-parametric Kruskal-Wallis' and Dunn's tests ($P\leq0.05$). These data wereexpressed as median [Q1-Q3]. All analyses were performed using Graph Pad Prism 5 (LaJolla, CA).

3. Results

3.1 Morphological analysis

The effects of mycotoxin treatments on ovarian morphology were assessed using a lesion score scale. The explants were observed microscopically and scored from 0 (no lesion) to 24. The incubation in culture medium (control group) resulted in scores rangingfrom 0 to 5 for all explants (Figure 1A). This group showed mild histological changes characterized by discrete disorganization of the granulosa cells layer and mild interstitialedema in the ovarian stroma (Figure 1B). The treatments with DON, FB1, and DON+FB1 induced a significant increase by 74.3%, 80.6%, and 81.9% in the lesion score comparedto control, respectively ($P<0.0001$). Lesion score was more pronounced in the explants exposed to FB1 and DON+FB1 compared to the individual DON treatment (Figure 1A) ($P<0.0001$). The explants exposed to DON revealed degenerated ovarian follicles with shrunken oocytes, showing deformity of the zona pellucida, cytoplasmic vacuolar degeneration, and nuclear pyknosis. Disorganized granulosa cells marked by the pyknotic nucleus and detachment from the basement membrane were also identified. Moreover, inthe ovarian stroma, DON induced a moderate presence of pyknotic cells and interstitial edema (Figure 1C). In the groups treated with FB1 or DON+FB1, histological changes were like those of the group exposed to DON, but the alterations in oocytes and ovarian stroma were more severe (Figure 1D and 1E).

Table 1 presents the effects of mycotoxins on follicular integrity. The three mycotoxin treatments induced a significant decrease in the number of normal follicles atall stages of development compared to control ($P\leq0.05$). The number of degenerated oocytes and granulosa cells (type II degeneration) tended to increase in primordial and primary follicles ($P\leq0.10$) in DON group. The explants exposed to FB1 and DON+ FB1 showed an increased number of primordial, primary, and growing follicles with type II degeneration compared to control ($P\leq0.05$). Furthermore, in samples exposed to individual or combined treatments, a reduction in the number of primordial and primary follicles with degeneration type I was observed compared to control ($P<0.01$).

Table 1. Number of normal and degenerated follicles according to the stages of follicular development (primordial to growing) in ovarian explants exposed to deoxynivalenol (DON), fumonisin (FB_1) and DON+FB_1 (Median [Q1-Q3] and mean percentage of follicles in each stage of follicular development and degeneration).

		Control	**DON**	**FB_1**	**DON+FB_1**
Primordial follicles	N	17.00a [5.0-24.00] (30.24%)	0.00b [0.00-1.25] (0.93%)	0.00b [0.00-1.25] (0.75%)	0.00b [0.00-0.75] (0.76%)
	TI	5.00a [4.00-9.50] (12.90%)	1.50ab [0.75-2.25] (2.78%)	0.00b [0.00-0.25] (0.25%)	0.00b [0.00-0.00] (0.00%)
	TII	1.00b [1.00-7.00] (6.85%)	17.00ab [14.00-44.00] (49.35%)	25.00a [20.00-42.25] (44.47%)	39.00a [20.00-46.50] (52.23%)
	TIII	1.00a [0.00-4.50] (4.03%)	0.00a [0.00-0.25] (0.31%)	0.00a [0.00-0.25] (0.25%)	0.00a [0.00-1.00] (1.01%)
Primary follicles	N	9.00a [6.50-12.00] (18.95%)	0.00b [0.00-1.00] (0.74%)	0.00b [0.00-0.50] (0.50%)	0.00b [0.00-0.00] (0.00%)
	TI	1.00a [0.50-3.00] (3.23%)	0.00ab [0.00-0.50] (0.62%)	0.00b [0.00-0.00] (0.00%)	0.00b [0.00-0.00] (0.00%)
	TII	1.00b [0.50-5.00] (4.84%)	13.50ab [9.00-15.00] (22.26%)	22.00a [6.00-36-00] (32.53%)	13.50a [10.25-17.50] (21.25%)
	TIII	0.00a [0.00-1.00] (0.81%)	0.50a [0.00-1.25] (1.24%)	0.00a [0.00-0.25] (0.25%)	0.00a [0.00-0.25] (0.25%)
Growing follicles	N	3.00a [2.00-4.50] (6.45%)	0.00b [0.00-0.25] (0.31%)	0.00b [0.00-0.50] (0.50%)	0.00b [0.00-0.00] (0.00%)
	TI	0.00a [0.00-1.00] (0.81%)	0.00a [0.00-0.00] (0.00%)	0.00a [0.00-0.00] (0.00%)	0.00a [0.00-0.00] (0.00%)
	TII	4.00b [1.50-7.00] (8.47%)	10.00ab [7.50-16.00] (21.15%)	14.50b [7.25-19.50] (20.52%)	16.00b [13.50-18.00] (23.99%)
	TIII	2.00a [0.00-2.00] (2.42%)	0.00a [0.00-0.25] (0.31%)	0.00a [0.00-0.00] (0.00%)	0.00a [0.00-1.00] (0.51%)
Total median		59.00a [26.50-75.00] -100%	45.00a [14.75-71.50] -100%	55.00a [28.75-76.13] -100%	63.00a [27.75-80.00] -100%

a,b Means within a row and type of degeneration followed by different letters indicate statistical significance. Kruskal-Wallis test followed by Dunn's tests ($P \leq 0.05$). Normal (N); type I degeneration (TI); type II degeneration (TII); type III degeneration (TIII).

3.2 Cell proliferation and apoptosis

Regarding granulosa cell proliferation of growing follicles, all treated groups (DON, FB_1, and DON+FB_1) showed a significant decrease ($P≤0.05$) in the number of NORs (2.5, 2.4, and 2.8-fold for DON, FB_1, and DON+FB_1, respectively) when compared to control (5.6) explants (Figure 2). Individual exposure of explants to DON and FB_1 induced no significant changes in cell apoptosis of primordial and primary follicles. However, a significant increase was observed in the number of apoptotic cells of growing follicles exposed to combined treatment of DON plus FB_1 compared to DON and control groups ($P≤0.05$) (Figure 2).

3.3 Evaluation of oxidative stress response

Oxidative stress consists of an imbalance between generation of free radicals and antioxidant defense capacity; therefore, both conditions must be analyzed. In this study, TBARS levels were used to evaluate lipid peroxidation, and GSH, FRAP and ABTS levels were associated with the antioxidant response.

Ovarian exposure to combined mycotoxins (DON+FB_1) induced a significant increase in GSH (64.78%; P= 0.03), ABTS (85.07%; P= 0.01), and FRAP (60.71%, P= 0.04), besides a decrease in TBARS levels (41.07%, P= 0.01) when compared to control explants (Figure 3). Similarly, the explants exposed to FB_1 showed an increase in ABTS (86.09%, P= 0.002) and a decrease in TBARS levels (41.31%, P= 0.01) (Figure 3). On the other hand, exposure to DON induced no significant difference in the oxidative stress response compared to control group (Figure 3).

4. Discussion

The maintenance of reproductive homeostasis is a complex process that frequently is disrupted by environmental factors [42]. In the last few years, a relationship between mycotoxin exposure and impaired fertility has been suggested [43, 44]. As mycotoxins contaminate (and multi-contaminate) up to 80% of grains worldwide [20], data on the effects and potential mechanisms of action of these products in the reproductive system are essential. In a previous study, we have shown DON toxicity in ovaries of pigs [30]. In this study, we have demonstrated that both DON and FB_1 induce significant changes in ovarian tissue in mono or multi-contamination exposure.

The effects of mycotoxins on ovarian tissue were mainly characterized by degenerated oocytes surrounded by disorganized granulosa cells and a pyknotic nucleus. Concerning follicular integrity, we observed that all mycotoxin treatments induced a reduction in the number of normal follicles for all follicular development stages. Similar results were observed previously in explants of ovaries exposed to DON [30]. In contrast, ovaries of pigs exposed to subchronic or chronic ingestion of DON-contaminated diets (3.4–19.1 mg/kg feed) showed no histological changes [45, 46].

Regarding FB_1 and DON + FB_1 exposure, an increase in primordial and growing follicles with type II degeneration (degeneration of both oocyte and granulosa cells) was noted, suggesting higher toxicity to FB_1 in comparison to DON in ovaries. In a previous study, rats fed an FB_1-contaminated diet (0.2, 10 and 20 mg/kg feed) for 14 days showed no histological abnormalities in their ovaries, but a significant reduction on the serum FSH and LH levels was observed [47].

The differences in experimental model (*in vivo* x culture tissue), animal species, exposure mode (*in vivo*/oral ingestion; *ex vivo*/direct exposure), and toxin concentration used here may have led to these divergent findings. The concentration of 100µM fumonisin B_1 used in this study corresponds to 72.18 mg/kg. Levels of fumonisins in natural contaminations are most often below 5 mg/kg; however, high levels (until 290 mg/kg) have been occasionally reported in feed samples [33]. Moreover, pigs are the animal species more susceptible to the effects of mycotoxins, and this may explain our different results [48].

Besides histological changes, we were also interested in the effects of these mycotoxins on cell proliferation and apoptosis. It has been demonstrated that complex interactions between oocytes and granulosa cells are essential for oocyte growth and maturation [49]. In this study, we showed that all mycotoxin treatments affect granulosa cell proliferation, resulting in a mean 2.5-fold decrease. This is in agreement with previous reports, wherein an inhibitory effect of DON and FB1 was observed against proliferation of porcine granulosa cells [23].

Interestingly, the exposure to multi-contamination treatment increased the number of apoptotic cells in growing follicles. In previous studies, caspase-3 expression in porcine granulosa cells and oocytes was not influenced by DON treatment [16, 17]. However, in porcine oocytes, DON induces an increase in the expression of autophagic-related genes (*Lamp2 and LC3*) and Annexin-5 (early apoptosis) [17]. Here, a similar effect was observed since no change in the expression of caspase-3 was found in the ovarian follicles of explants treated with DON. No data were found regarding FB1 exposure and apoptosis of oocytes or granulosa cells. However, it is known that, in esophageal cancer cells, the effect of fumonisin B1 on apoptosis is dose-dependent [50]. Once the MAPK pathway is essential for oocyte maturation [51], and DON affects such a pathway [17], we can hypothesize that the co-exposure acts in a synergistic way to induce apoptosis in granulosa cells.

Since oxidative stress has been considered an important mechanism of toxicity of mycotoxins in different cells and systems models [52], we have evaluated the effect of DON and/ plus FB1 on the antioxidant potential and lipoperoxidation in the ovarian explants. No change in the antioxidant status and TBARS levels were observed in the samples exposed to DON. But, according to a recent study of Lan et al. [53], in mice oocytes, the oxidative stress might be a mechanism of DON toxicity. As the complex patterns of cellular differentiation seen *in vivo* are maintained in the explant model, different results can be obtained from that observed with isolated single cell types. We also noted an increase in the antioxidant status and a decrease in the TBARS levels in the samples exposed to FB_1 and DON+FB_1. In the ovaries, ROS are produced mainly by endothelial cells and resident leucocytes localized around preovulatory follicles or infiltrated between the granulosa cells [54]. Moreover, the production of ROS has been identified in steroidogenic cells during the conversion of cholesterol into pregnenolone via cytochrome P450 [55]. Accumulating data have shown that ROS can play animportant physiological role in the ovaries [56, 57]. In a previous study, the exposure ofoocytes-cumulus complexes of rats to antioxidants reversibly inhibited germinal vesicle breakdown [58]. Besides, antioxidants and neutrophil-depleting monoclonal antibody significantly reduced the ovulation rate in *in vitro* and *in vivo* animal models [57, 59, 60].Based on these data, the increased antioxidant potential induced by treatments containing FB_1 may affect the reproductive capacity in pigs.

5. Conclusions

Continuous ingestion of low doses of mycotoxins is a reality for humans and animals, but its impact on the reproductive system is scarcely known. In the present study, we have demonstrated that DON and/plus FB_1 exposure induced histological changes in the ovaries of pigs, including alterations in cell proliferation and apoptosis. A modulationof the oxidative stress response was also detected, which might be affecting the ovulation process. In this context, future studies should be designed to characterize the role of ROS in the reproductive toxicity of mycotoxins in an *in vivo* model. Given the histophysiological similarity between pig and human ovaries, future studies shouldinvestigate the possible role of mycotoxins on female infertility.

Declaration of interest

The authors state no conflict of interest.

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6. References

[1] Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. PLOS Medicine 2012;9:e1001356.

[2] Sun H, Gong T-T, Jiang Y-T, Zhang S, Zhao Y-H, Wu Q-J. Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990–2017: results from a global burden of disease study, 2017. Aging (Albany NY) 2019;11:10952-91.

[3] Marques-Pinto A, Carvalho D. Human infertility: are endocrine disruptors to blame? Endocr Connect 2013;2:15-29.

[4] Demaegdt H, Daminet B, Evrard A, Scippo M-L, Muller M, Pussemier L, et al. Endocrine activity of mycotoxins and mycotoxin mixtures. Food and Chemical Toxicology 2016;96:107-16.

[5] Milićević DR, Škrinjar M, Baltić T. Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. Toxins 2010;2:572-92.

[6] Fink- Grernmels J. Mycotoxins: their implications for human and animal health. Veterinary Quarterly 1999;21:115-20.

[7] Cortinovis C, Pizzo F, Spicer LJ, Caloni F. Fusarium mycotoxins: Effects on reproductive function in domestic animals—A review. Theriogenology 2013;80:557-64.

[8] Santos R, Schoevers E, Roelen B, Fink-Gremmels J. Mycotoxins and female reproduction: in vitro approaches. World mycotoxin journal 2013;6:245-53.

[9] Urbanek KA, Habrowska- Górczyńska DE, Kowalska K, Stańczyk A, Domińska K, Piastowska- Ciesielska AW. Deoxynivalenol as potential modulator of human steroidogenesis. Journal of Applied Toxicology 2018;38:1450-9.

[10] Shifrin VI, Anderson P. Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J Biol Chem 1999;274:13985-92.

[11] Diaz-Llano G, Smith T. Effects of feeding grains naturally contaminated with Fusarium mycotoxins with and without a polymeric glucomannan mycotoxin adsorbent on reproductive performance and serum chemistry of pregnant gilts. Journal of Animal Science 2006;84:2361-6.

[12] Diaz-Llano G, Smith T. The effects of feeding grains naturally contaminated with Fusarium mycotoxins with and without a polymeric glucomannan adsorbent on lactation, serum chemistry, and reproductive performance after weaning of first-parity lactating sows. Journal of Animal Science 2007;85:1412-23.

[13] Malekinejad H, Schoevers EJ, Daemen IJ, Zijlstra C, Colenbrander B, Fink-Gremmels J, et al. Exposure of oocytes to the Fusarium toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. Biology of reproduction 2007;77:840-7.

[14] Schoevers E, Fink-Gremmels J, Colenbrander B, Roelen B. Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. Theriogenology 2010;74:968-78.

[15] Lu Y, Zhang Y, Liu J-Q, Zou P, Jia L, Su Y-T, et al. Comparison of the toxic effects of different mycotoxins on porcine and mouse oocyte meiosis. PeerJ 2018;6:e5111.

[16] Medvedova M, Kolesarova A, Capcarova M, Labuda R, Sirotkin AV, Kovacik J, etal. The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells in vitro. Journal of Environmental Science and Health Part B 2011;46:213-9.

[17] Han J, Wang Q-C, Zhu C-C, Liu J, Zhang Y, Cui X-S, et al. Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during

porcine oocyte maturation. Toxicology and applied pharmacology 2016;300:70-6.

[18] Capcarova M, Kolesarova A, Medvedova M, Petruska P, Sirotkin AV. Induction of Hsp70 and antioxidant status in porcine granulosa cells in response to deoxynivalenol and zearalenone exposure in vitro. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 2014;8:244-9.

[19] Mishra S, Dwivedi PD, Pandey HP, Das M. Role of oxidative stress in Deoxynivalenol induced toxicity. Food and Chemical Toxicology 2014;72:20-9.

[20] Eskola M, Kos G, Elliott CT, Hajšlová J, Mayar S, Krska R. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate'of 25%. Crit Rev Food Sci Nutr 2019;3:1-17.

[21] Voss K, Smith G, Haschek W. Fumonisins: toxicokinetics, mechanism of action and toxicity. Animal Feed Science and Technology 2007;137:299-325.

[22] Wang X, Wu Q, Wan D, Liu Q, Chen D, Liu Z, et al. Fumonisins: oxidative stress-mediated toxicity and metabolism in vivo and in vitro. Archives of Toxicology 2016;90:81-101.

[23] Cortinovis C, Caloni F, Schreiber NB, Spicer LJ. Effects of fumonisin B1 alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation and steroid production. Theriogenology 2014;81:1042-9.

[24] Gbore F. Growth performance and puberty attainment in growing pigs fed dietary fumonisin B1. Journal of animal physiology and animal nutrition 2009;93:761-7.

[25] Albonico M, Schutz LF, Caloni F, Cortinovis C, Spicer LJ. In vitro effects of the Fusarium mycotoxins fumonisin B1 and beauvericin on bovine granulosa cell proliferation and steroid production. Toxicon 2017;128:38-45.

[26] Fujimoto VY, Bloom MS. Role of environmental factors and gonadotoxin exposure in unexplained female infertility. Unexplained Infertility: Springer, New York, NY; 2015. p. 161-73.

[27] Wang X, Zhang J, Xu W, Huang Q, Liu L, Tian M, et al. Low-level environmental arsenic exposure correlates with unexplained male infertility risk. Science of the Total Environment 2016;571:307-13.

[28] Choy CM, Lam CW, Cheung LT, Briton- Jones CM, Cheung L, Haines CJ. Infertility, blood mercury concentrations and dietary seafood consumption: a case–control study. BJOG: An International Journal of Obstetrics & Gynaecology 2002;109:1121-5.

[29] Bracarense APF, Pierron A, Pinton P, Gerez JR, Schatzmayr G, Moll W-D, et al. Reduced toxicity of 3-epi-deoxynivalenol and de-epoxy-deoxynivalenol through deoxynivalenol bacterial biotransformation: In vivo analysis in piglets. Food and Chemical Toxicology 2020;140:111241.

[30] Gerez JR, Desto SS, Bracarense APFRL. Deoxynivalenol induces toxic effects in the ovaries of pigs: an ex vivo approach. Theriogenology 2017;90:94-100.

[31] Basso K, Gomes F, Bracarense APL. Deoxynivanelol and fumonisin, alone or in combination, induce changes on intestinal junction complexes and in E-cadherin expression. Toxins 2013;5:2341-52.

[32] Grenier B, Loureiro- Bracarense AP, Lucioli J, Pacheco GD, Cossalter AM, Moll WD, et al. Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. Molecular nutrition & food research 2011;55:761-71.

[33] Gruber-Dorninger C, Jenkins T, Schatzmayr G. Global mycotoxin occurrence in feed: A ten-year survey. Toxins 2019;11:375.

[34] Knutsen HK, Alexander J, Barregård L, Bignami M, Brüschweiler B, Ceccatelli S, et al. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA J 15 2017;15:e04718.

[35] Andrade ER, Seneda MM, Alfieri AA, de Oliveira JA, Bracarense APFRL, Figueiredo JR, et al. Interactions of indole acetic acid with EGF and FSH in the culture of ovine preantral follicles. Theriogenology 2005;64:1104-13.

[36]Bizarro-Silva C, Santos MM, Gerez JR, González SM, Lisboa LA, Seneda MM. Influence of follicle-stimulating hormone concentrations on the integrity and development of bovine follicles cultured in vitro. Zygote (Cambridge, England) 2018;26:417-23.

[37] Phoophitphong D, Wangnaitham S, Srisuwatanasagul S, Tummaruk P. The use of proliferating cell nuclear antigen (PCNA) immuno-staining technique to determine number and type of follicles in the gilt ovary. Livest Sci 2012;150:425-31.

[38] Crocker J, Skilbeck N. Nucleolar organiser region associated proteins in cutaneous melanotic lesions: a quantitative study. J Clin Pathol 1987;40:885-9.

[39] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25:192-205.

[40] Katalinic V, Modun D, Music I, Boban M. Gender differences in antioxidant capacity of rat tissues determined by 2, 2′-azinobis (3-ethylbenzothiazoline 6-sulfonate;ABTS) and ferric reducing antioxidant power (FRAP) assays. Comp Biochem Physiol C Toxicol Pharmacol 2005;140:47-52.

[41] Guedes RP, Dal Bosco L, Teixeira CM, Araújo AS, Llesuy S, Belló-Klein A, et al. Neuropathic pain modifies antioxidant activity in rat spinal cord. Neurochem Res 2006;31:603-9.

[42] Mochizuki K, Hariya N, Honma K, Goda T. Relationship between epigenetic regulation, dietary habits, and the developmental origins of health and disease theory. Congenit Anom (Kyoto) 2017;57:184-90.

[43] El Khoury D, Fayjaloun S, Nassar M, Sahakian J, Aad PY. Updates on the Effect of Mycotoxins on Male Reproductive Efficiency in Mammals. Toxins 2019;11:515.

[44] Eze UA, Okonofua FE. High prevalence of male infertility in Africa: are mycotoxins to blame? Afr J Reprod Health 2015;19:9-17.

[45] Friend D, Trenholm H, Fiser P, Hartin K, Thompson B. Effect of feeding diets containing deoxynivalenol (vomitoxin)-contaminated wheat or corn on the feed consumption, weight gain, organ weight and sexual development of male and female pigs. Canadian Journal of Animal Science 1986;66:765-75.

[46] Trenholm H, Thompson B, Foster B, Charmley L, Hartin K, Coppock R, et al. Effects of feeding diets containing Fusarium (naturally) contaminated wheat or pure deoxynivalenol (DON) in growing pigs. Canadian Journal of Animal Science 1994;74:361-9.

[47] Gbore FA, Owolawi TJ, Erhunwunsee M. Evaluation of the reproductive toxicityof dietary fumonisin B1 in rats. Jordan Journal of Biological Sciences 2012;147:1-7.

[48] Bertero A, Moretti A, Spicer LJ, Caloni F. Fusarium molds and mycotoxins: Potential species-specific effects. Toxins 2018;10:244.

[49] Orisaka M, Tajima K, Tsang BK, Kotsuji F. Oocyte-granulosa-theca cell interactions during preantral follicular development. J Ovarian Res 2009;2:1-9.

[50] Khan R, Phulukdaree A, Chuturgoon A. Concentration-dependent effect of fumonisin B1 on apoptosis in oesophageal cancer cells. Hum Exp Toxicol 2018;37:762-71.

[51] Fan H-Y, Sun Q-Y. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biol Reprod 2004;70:535-47.

[52] Silva E, Bracarense A, Oswald I. Mycotoxins and oxidative stress: where are we? World Mycotoxin J 2018;11:113-34.

[53] Lan M, Han J, Pan MH, Wan X, Pan ZN, Sun SC. Melatonin protects against defects induced by deoxynivalenol during mouse oocyte maturation. J Pineal Res 2018;65:e12477.

[54] Kodaman PH, Behrman HR. Endocrine-regulated and protein kinase C-dependent generation of superoxide by rat preovulatory follicles. Endocrinology 2001;142:687-93.

[55]Hanukoglu I. Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. Drug Metab Rev 2006;38:171-96.

[56] Rizzo A, Roscino M, Binetti F, Sciorsci R. Roles of reactive oxygen species in female reproduction. Reprod Dom Anim 2012;47:344-52.
[57] Sugino N. Reactive oxygen species in ovarian physiology. Reprod Med Biol 2005;4:31-44.
[58] Takami M, Preston S, Toyloy V, Behrman HR. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. Am J Physiol 1999;276:684-8.
[59] Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N. Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A 2011;108:1462-7.
[60] Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reproductive biology and endocrinology 2005;3:1-10.

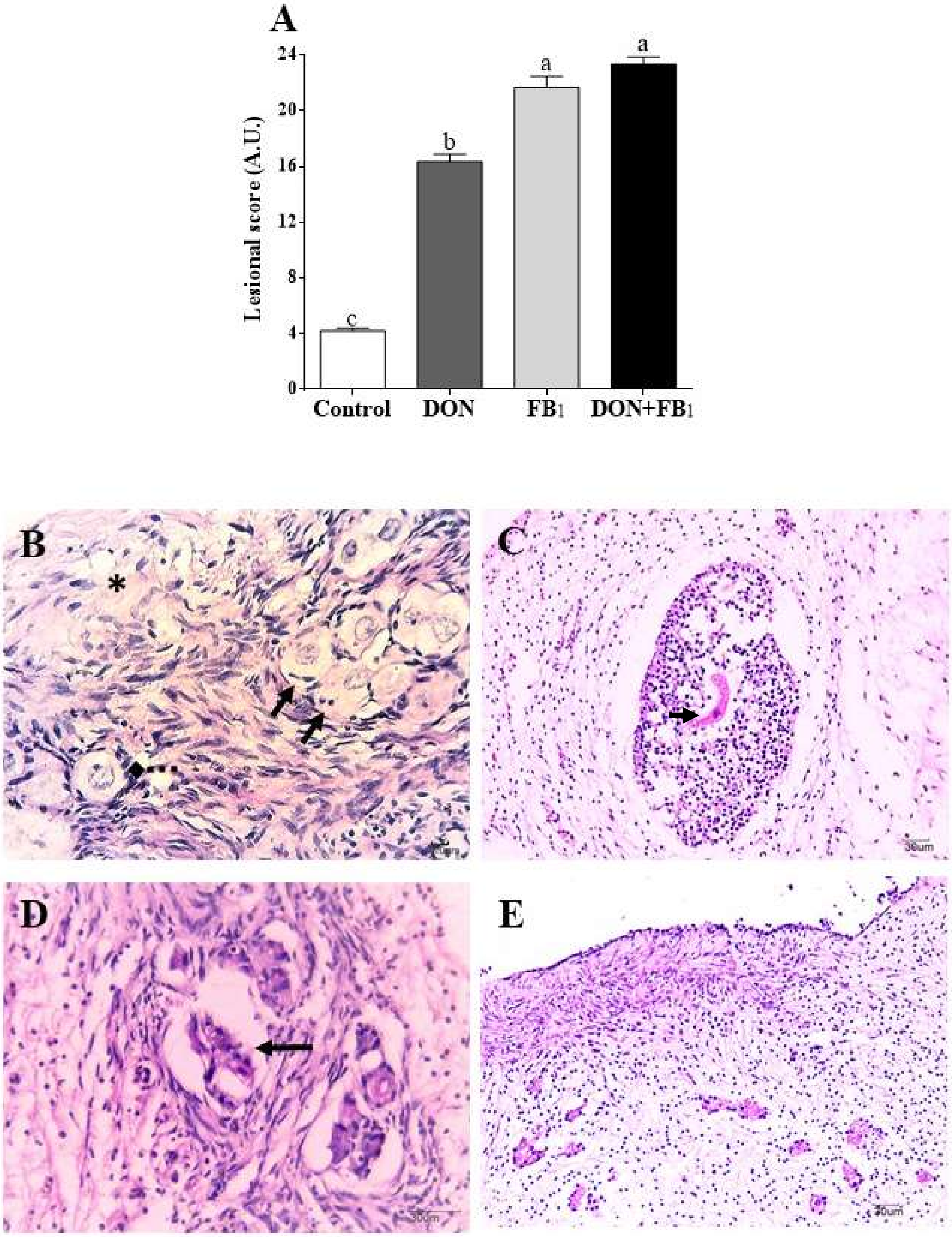

Figure 1. Morphology of pigs' ovarian explants exposed to treatments: control (medium with DMEM) (□); deoxynivalenol (10μM of DON) (■); fumonisin B_1 (100μM of FB_1) (▨); DON+FB_1 (10μM+100μM) (■). (A) The lesion score of ovarian explants exposed to different treatments. Values are mean±standard error of the mean. a, b scripts are different at $P < 0.0001$ by Tukey's test. (B) Control group: mild interstitial edema in the ovarian stroma (*) with primordial follicles showing normal morphology (➔) or discrete disorganization of the granulosa cells layer (━◆), HE, Bar 50 μm. (C) DON group: growing follicles with disorganization of granulosa cells and oocyte with flattening and pyknotic nucleus (➔), HE, Bar 30 μm. (D) FB_1 group: degeneration of primordial follicles showing pyknotic nuclei in granulosa cells (arrow) and absence of oocyte, HE, Bar 30 μm. (E) DON+FB_1 group: loss of normal stromal morphology and severe interstitial edema, HE, Bar 30 μm.

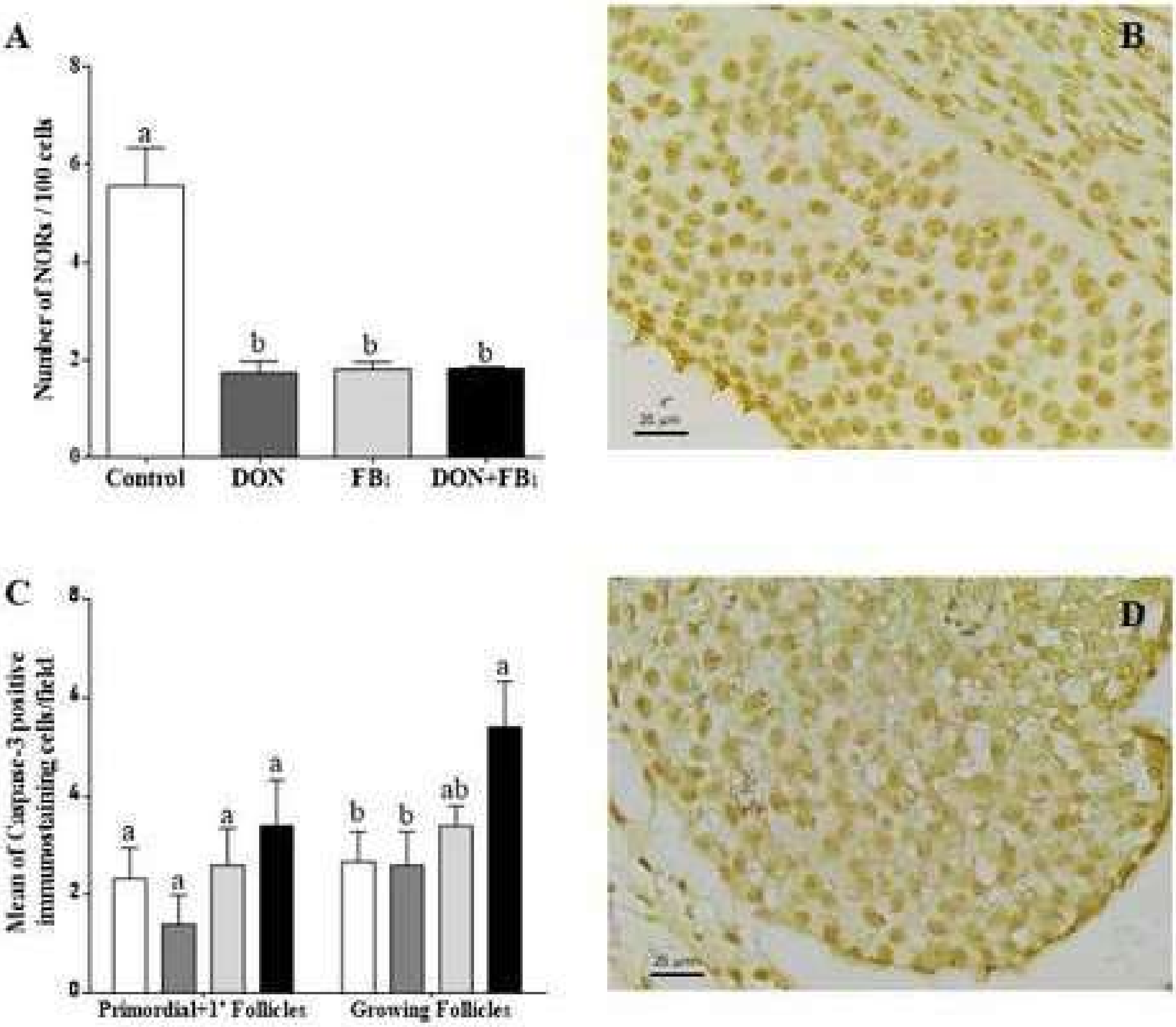

Figure 2. Effects of different treatments in the proliferation and apoptosis of granulosa cells. (A) Mean number of NORs per granulosa cell in growing follicles. (B) Control group. Ovaries of pigs showing growing follicles with normal number of NORs. AgNOR method. Bar 25 µm. (C) Number of Caspase-3 immunostained cells in the primordial and primary (1°) follicles, and growing follicles. (D) DON+FB$_1$ group showing a reduced number of NORs. AgNOR method. Bar 25 µm. Treatments: control group (MEM culture medium) (□); DON (10µM) (■); FB$_1$ (100µM FB$_1$) (▨); DON+ FB$_1$ (10µM + 100µM) (■). Values are mean±standard error of the mean. a, b scripts are different at P ≤ 0.05 by non-parametric Kruskal-Wallis' and Dunn's tests (A) or Tukey's test (C).

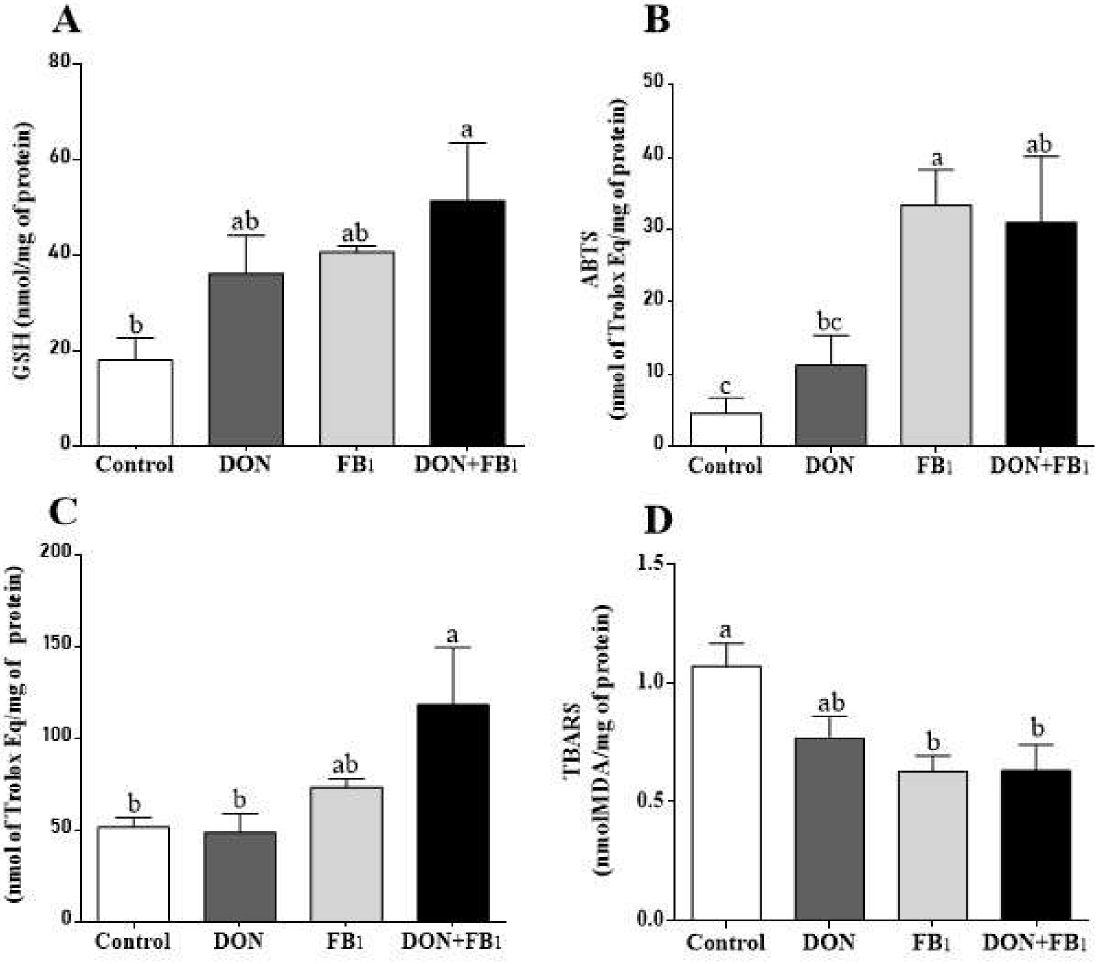

Figure 3. Effects of different treatments in the oxidative stress response in ovarian explants from pigs. Control (MEM culture medium) (□); DON (10µM of deoxynivalenol) (■); FB_1 (100µM FB_1) (▒); DON+FB_1 (10µM+100µM) (■). (A) GSH; (B) ABTS; (C) FRAP; (D) TBARS. Values are mean±standard error of the mean. a, b scripts are different at $P < 0.05$ by Tukey's test.

Credit Author Statement

Juliana R. Gerez and Ana Paula F. R. L. Bracarense delineated the experiment and wrote the manuscript. Thaynara Camacho, Victor Hugo Marutani and Ricardo Luís N. de Matos performed the histological and immunohistochemical assays. Miriam S. N. Hohmann performed the oxidative stress analysis. Waldiceu A. Verri Jr. made a critical review of the manuscript.

4.2 Effects of deoxynivalenol exposure at peripuberty over testicles of rats: structural and functional alterations

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Abstract

Deoxynivalenol (DON) is related to reduced reproductive performance in males and females in several species. Children and adolescents showed a high risk of exposure to DON, however, no study has evaluated reproductive effects of DON at peripubertal. The present study aimed to evaluate the effects of DON at peripuberty on the testicles of pubertal rats. To achieve this, 10 Wistar rats (28-day-old) were fed for 28 days with a DON-contaminated diet (9.4 mg/kg) or a control diet. After the experimental period, rats (56-day-old) were euthanized and the following evaluations were performed in the testicles: dynamics of spermatogenesis, tubular morphometry, number of Sertoli cells and Leydig cells, analysis of caspase-3 expression, and the index of cell proliferation using the nucleolus organizing regions (NOR) method. Ingestion of DON-contaminated diet induced a significant reduction in the number of Sertoli and Leydig cells and the number of seminiferous tubules in stage XIV. A significant increase in the number of NORs in seminiferous tubules in stage I-VI was observed in animals receiving the DON diet. No significant difference was noted in tubular morphometry or caspase-3 expression. Taken together, our results unraveled that the peripubertal exposure to DON compromised the testicular structure of pubertal rats, changing the dynamics of spermatogenesis.

Keywords: spermatogenesis, mycotoxins, reproductive toxicity.

Introduction

With the activation of the hypothalamic-pituitary-gonadal axis in puberty, a complex series of physiological changes lead to reproductive maturity. Accordingly, puberty itself is a large window of vulnerability to toxic agents which may result in temporary or permanent reproductive damage in adulthood (Diamanti-Kandaralis and Gore, 2011). Among all food contaminants, mycotoxins contamination of cereals has now become a major issue in most parts of the world because of its toxic effects on animals and humans (Urbanek *et al.*, 2018). Mycotoxins result from the secondary metabolism of mycotoxigenic fungi that can contaminate grains during planting, harvesting, and storage (Bennett and Klich, 2003). They are highly resistant to remain in the food even after processing methods (Mishra *et al.*, 2014). Mycotoxins cause a variety of adverse health effects in humans and animals by affecting mainly the digestive, immune, and reproductive systems. However, whether exposure to these toxins at puberty can induce reproductive damage is a hypothesis that has been poorly evaluated.

One of the most prevalent mycotoxins in temperate climates is deoxynivalenol (DON), which has a worldwide incidence of 59% in grains and cereals, however, it can occur in higher concentrations in the European and Asian continents (Lee and Ryu, 2017). DON is classified in the trichothecenes group (McCormick *et al.,* 2011), inducing a ribotoxic effect that results in the inhibition of protein and nucleic acid synthesis (Pestka *et al.,* 2005). The susceptibility to toxicity varies according to the animal species, with swine being the most sensitive, followed by rodents (Pestka, 2007). Acute exposure can cause gastroenteritis with vomiting, leukocytosis, and hemorrhage, while in cases of chronic ingestion there will be changes in the immune system, anorexia, and decreased nutritional efficiency (Pestka and Smolinski, 2005). Furthermore, in the past few years, a relationship between DON exposure and impaired fertility has been suggested (Eze *et al.,* 2018). However, few studies are approaching the effects of DON on the male reproductive system (Khoury *et al.,* 2019).

In adult rats, DON induces a reduction in the spermatid and sperm number with an increase of degenerated germ cells and abnormal sperm. Moreover, DON affects the steroidogenesis increasing the release of follicle-stimulating hormone and luteinizing hormone and reducing the testosterone serum level (Sprando *et al.,* 2005). Additionally, spermatogenesis disorder and testicular damage were observed in adult mice exposed to DON. Also, DON affects the blood- testis barrier (BHB) of mice decreasing the junction proteins expression as occludin, connexin-43, and N-cadherin (Cao *et al.,* 2020). Nevertheless, the reproductive effects of DON at puberty have been overlooked until the present time. Therefore, the present study was undertaken to explore peripubertal exposure to DON on the reproductive parameters of male rats through histological, histochemical, morphometric, and immunohistochemical analysis.

Material and Methods

Animals and experimental conditions

The study was approved by the Ethics Committee on the Use of Animals (CEUA n° 6986.2017.27). Ten male Wistar rats (*Rattus norvegicus*), 28 days old were used in this study.

The animals were housed in micro-insulated polycarbonate boxes measuring 41x34x16 cm, with a stainless-steel wire mesh, feeder, and drinking bowl. The bed was made of pine wood shavings, of good quality, and autoclaved. The experiment followed the Animal Research Ethical Principles of the Brazilian College of Animal Experimentation with a standard diet (Quimtia®), water ad libitum, adequate temperature (± 22 °C), ventilation, humidity (40-70%), and photoperiod (12 hours of light: 12 hours of dark). The diet was contaminated with a crude extract of DON (40 mg/kg) produced at the Laboratory of Mycology, Luiz de Queiroz College of Agriculture, Universidade de São Paulo. A blend (standard diet+DON) was prepared at Universidade Estadual de Londrina facilities using a commercial feed mixer. The diets were sent to the Samitec laboratory (Santa Maria – RS/Brazil), where the mycotoxins levels were assessed using the HPLC/MS-MS method. The control diet showed lower levels of DON than the limit of quantification established (200 µg/kg with a stated accuracy of 99%).

Experimental design

The animals were divided into two experimental groups (control and DON) in a completely randomized design. For 28 days, control animals received a mycotoxin-free diet and the treated group received a diet contaminated with DON (9.4 mg/kg of feed). According to Grenier and Applegate (2013), the dose of DON used in the treatment represents a dose occasionally found in commodities, feeds, and feed ingredients. However, a realistic dose was not chosen since a greater metabolism of DON to DOM-1 is found in murine when compared to humans (Lattanzio *et al.,* 2011). In addition, this dose agrees with other relevant studies that have evaluated the toxicity of DON in the murine species (Bracarense *et al.,* 2017; Clark *et al.,* 2015). Thus, at the end of the pubertal period (Ojeda *et al.,* 1980), animals of 56 days old were euthanized using an overdose of sodium pentobarbital intraperitoneally. Then, the testicles were removed and fixed for 24 hours in a 10% formaldehyde buffered solution. Promptly, they were processed according to the histological routine and stained in hematoxylin and eosin (HE). In addition, the silver staining technique for argyrophilic proteins related to the nucleolus organizing regions (AgNOR) adapted from Chacur and collaborators (2015) was performed.

Testicle sections were also processed for immunohistochemical analysis to assess the apoptosis index through the expression of caspase-3. Antigenic recovery was performed in a pressure cooker using citrate buffer pH 6.0. The sections were incubated for 22 hours in a humid chamber at 4°C with the primary anti-cleaved-caspase-3 antibody (CCasp3) (anti-ASP175, dilution 1:200, Cell Signaling Technology, Beverly, MA, EUA). Subsequently, they were incubated with a secondary antibody bound to the polymer (Polydector) (Super PictureTM Polymer Detection Kit, Invitrogen Corporation, Camarillo, CA, EUA) for 30 minutes. The final reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB), the sections were stained with Harris hematoxylin and mounted with a coverslip and synthetic resin. To control the reaction, histological sections of lymph nodes were used, following the manufacturer's recommendations.

Zootechnical parameters and testicular weight

During the experiment, the animals were weighed weekly and body weight gain and feed intake were calculated for each week. In addition, after euthanasia, the testicles were carefully collected and weighed.

Testicular analysis

The dynamics of spermatogenesis (Figure S1) were performed in 100 random tubular sections per animal that were classified into four categories: stages I-VI, VII-VIII, IX-XIII, and XIV of the seminiferous epithelium cycles (Leblond and Clermont, 1952), according to Siervo et al. (2015).

Cell proliferation was assessed using the AgNOR technique adapted from Chacur and collaborators (2015). The quantification of the nucleolus organizing regions (NORs) was performed randomly in five tubules from each stage of the spermatogenesis dynamics (except stage XIV due to the impossibility of locating meiosis II of spermatocytes II, characteristic of this stage). In each tubule, the number of NORs in 20 cells was quantified, totaling 100 cells per stage.

For morphometric analysis, the height of the epithelium and the diameter of the seminiferous tubules were evaluated. The analysis was performed in phase IX tubules of each testicle. In each seminiferous tubule, four height measurements and four diameter measurements were obtained to perform averages and statistical analysis (Favareto *et al.,* 2011). The analyzes were performed using a Leica optical microscope with a MOTIC plus imaging system (Richmond, Canada).

To evaluate the cell apoptosis index in seminiferous tubules, immunohistochemistry was used to assess the expression of caspase-3. The analysis was performed on 300 seminiferous tubules that were classified as immunostained or not. Tubules immunostained were subsequently subjected to the same classification used in the dynamics of spermatogenesis (Gonçalves *et al.,* 2017).

The number of Leydig cells was counted in 10 random fields of interstitial tissue per animal using the 40x objective and a MOTIC plus imaging system (Richmond, Canada) (Guerra *et al.,* 2017). The counting of the number of Sertoli cells was determined in 20 cross-sections of seminiferous tubules in phase VII-VIII for each testicle with the aid of the 40x objective (Nassr *et al.,* 2010).

Statistical analysis

Statistical analysis was performed after checking the assumptions of normality (Shapiro test) and homogeneity (Bartlett's test). The data of the different variables were submitted to a Student's t-test with a significance level of 0.05.

Results

Zootechnical parameters and testicular weight

After the 2nd, 3rd, and 4th week a significant reduction of the feed intake (10.71%, 16.20 %, and 19.34%) was observed in the animals challenged with DON (Figure 1A). Similar results were observed weekly on the weight gain of animals, where a significant reduction of 34.54%, 27.58%, 27.77%, and 15.55% was observed, respectively (Figure 1B). In addition, no difference was observed between the testicular weight of control and challenged animals (data not shown).

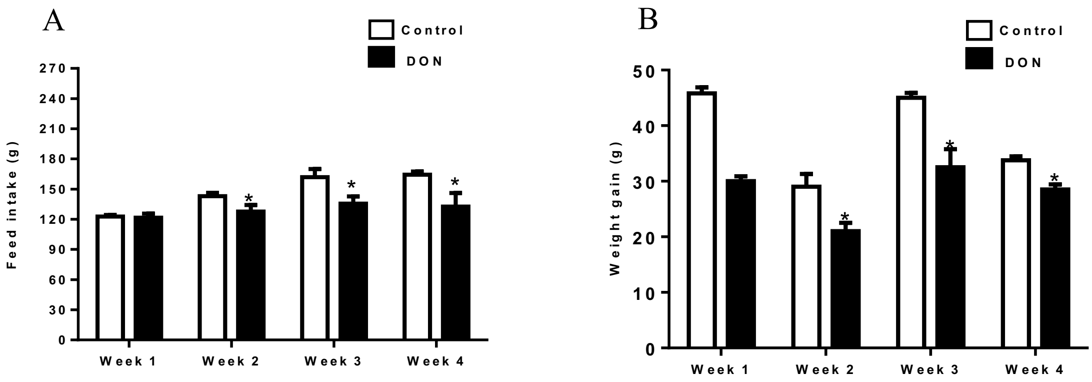

Figure 1. Effect of peripubertal exposure to deoxynivalenol (DON) on the zootechnical parameters of experimental animals. A) Weekly feed intake of experimental animals. B) Weekly weight gain of experimental animals. Experimental groups: (□) mycotoxin-free control diet or (■) contaminated diet with 9.4 mg/kg DON of feed from the postnatal day 28 to 56. Values are expressed as mean ± S.E.M. * indicates a statistically significant difference using the Student's t-test, $p < 0.05$.

Dynamics of spermatogenesis and tubular morphometry

The percentage of seminiferous tubules in stage XIV per testicle was 1.5 in the control and 0.25 in the group treated with DON. Thus, there was a significant reduction (83.33%) in 23 seminiferous tubules at this stage ($p = 0.007$) in animals challenged with DON. However, the other stages of sperm kinetics show no significant changes (Table 1). The results of the tubular morphometry are shown in Table 1. There was no significant difference in the parameters evaluated between the groups.

Table 1. Diameter of seminiferous tubules, height of the germinal epithelium, and dynamics of spermatogenesis in rats exposed to a diet contaminated by 9.4 mg/kg DON of feed from the postnatal day 28 to 56

	Control	**DON**
Morphometry (µm)		
Diameter of seminiferous tubules	258.31 ±4.61	245.77 ±6.11
Germinative epithelium height	76.01 ±3.36	71.08 ±3.86
Dynamics of spermatogenesis (%)		
I – VI	26.00 ±2.72	24.00 ±2.28
VII – VIII	62.50 ±2.54	66.00 ±1.45
IX – XIII	10.00 ±0.63	9.75 ±1.28
XIV	1.5 ±0.43	0.25 ±0.19*

Values are expressed as mean ± S.E.M. * indicates a statistically significant difference using the Student's t-test, $p < 0.05$.

AgNOR evaluation

The control group and DON showed the average of NORs in stages I-VI of 1.6 and 2.0, respectively. In these stages, a significant increase in the number of NORs (26,58%) was observed in animals receiving the contaminated diet ($p = 0.002$). On the other hand, no difference between the groups was verified in stages VII-VIII and IX-XIII (Figure 2).

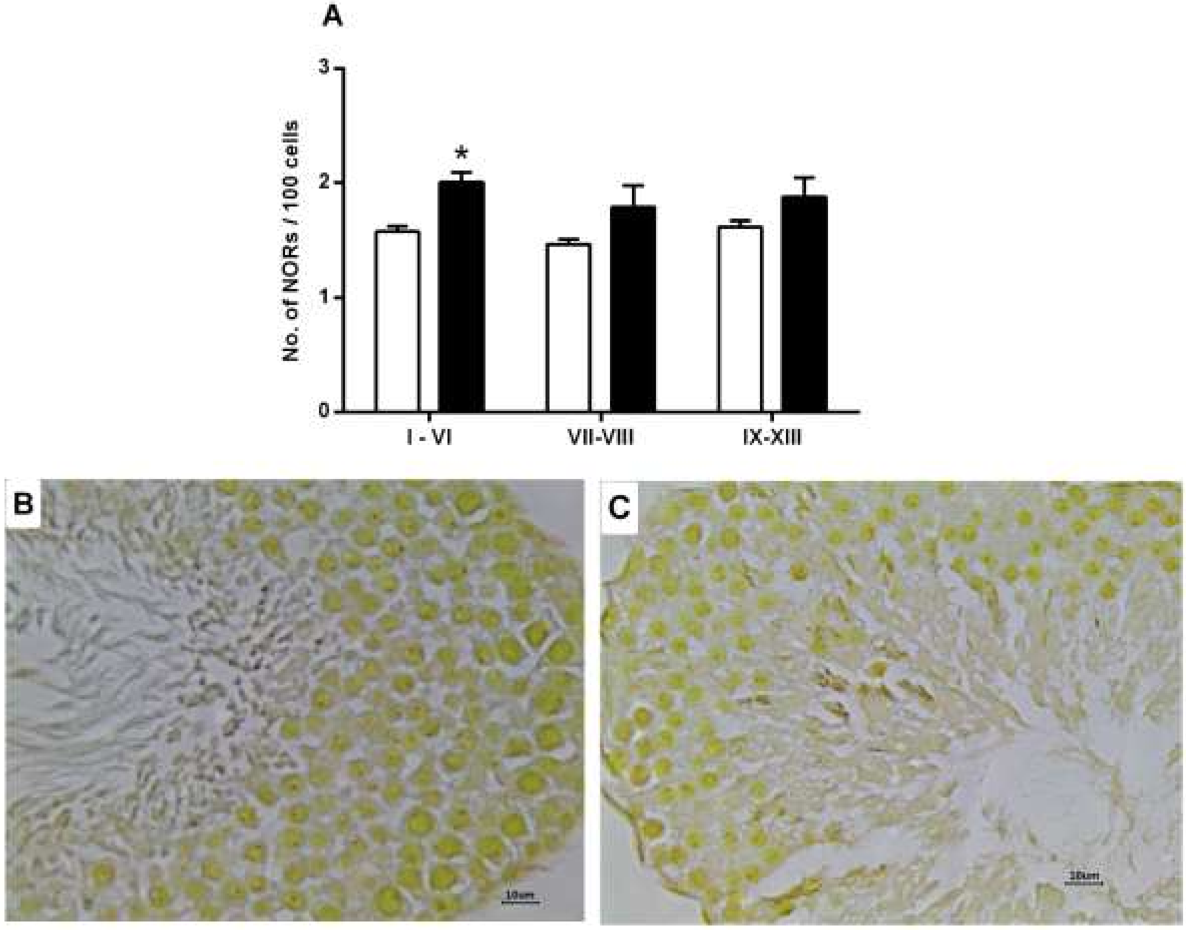

Figure 2. Effect of peripubertal exposure to deoxynivalenol (DON) on the proliferation of germ cells of the seminiferous epithelium according to the different stages of spermatogenic kinetics. A) Number of NORs (nucleolus organizing regions) present in 100 cells from each stage of sperm kinetics. Experimental groups: (□) mycotoxin-free control diet or (■) contaminated diet with 9.4 mg of DON/kg of feed from the postnatal day 28 to 56. Values are expressed as mean ± S.E.M. * indicates a statistically significant difference using the Student's t test, $p < 0.05$. B) Seminiferous tubules of control animals. Germ cells with nucleolus organizing regions. C) Seminiferous tubules of animals exposed to DON. Germ cells with nucleolus organizing regions. AgNOR method. 10 µm bar.

Histological and immunohistochemical analysis

The effect of DON in the number of Leydig and Sertoli cells was evaluated through histological analysis. The average number of Leydig cells per field was 21.7 and 18.3 in the control and DON groups, respectively (Figure 3A). There was a significant reduction ($p = 0.01$) in the number of Leydig cells in animals exposed to DON when compared to the control. The average number of Sertoli cells per tubule was 20.7 and 18.9 in the control and DON groups, respectively (Figure 3B). Sertoli cells number showed a significant decrease ($p = 0.02$) compared to the control.

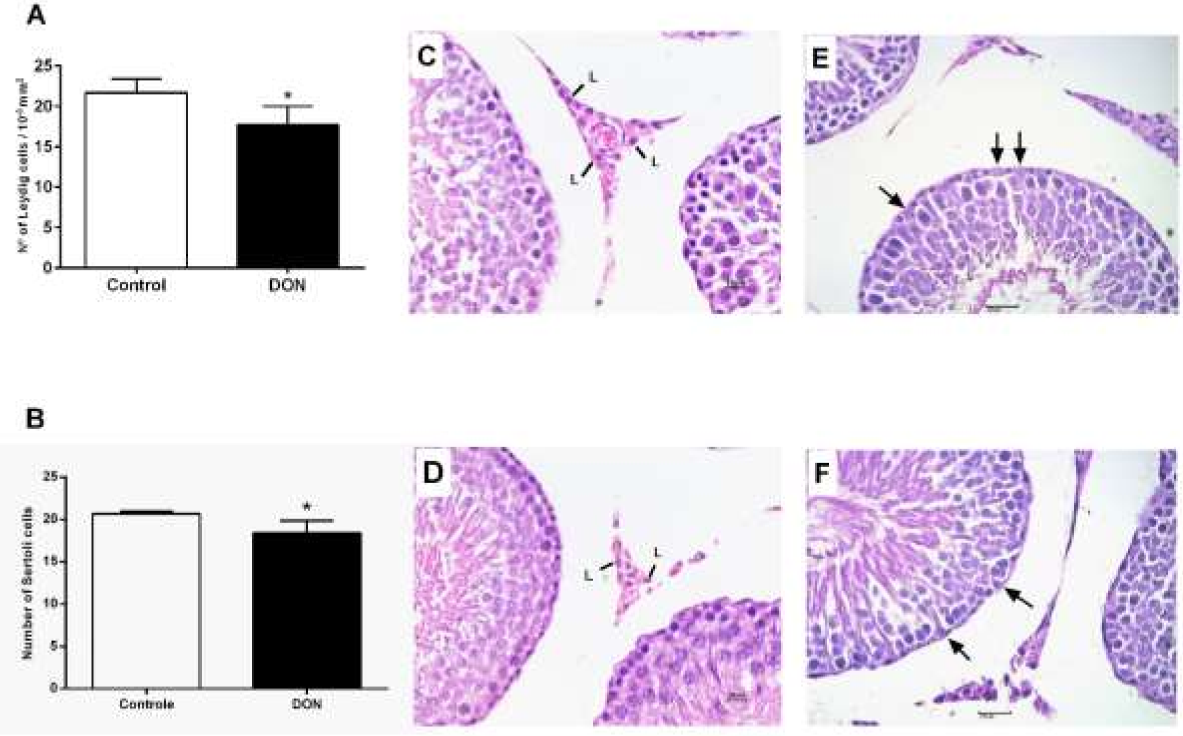

Figure 3. Effect of peripubertal exposure to deoxynivalenol (DON) on the number of Leydig cells (L), and Sertoli cells (arrow) in the testicles of rats exposed to a (□) mycotoxin-free or (■) control diet contaminated with 9.4 mg/kg DON of feed from postnatal day 28 to 56. A) Number of Leydig cells present in 10^{-3} mm^2. B) Number of Sertoli cells per seminiferous tubules. Values are expressed as mean ± S.E.M. * indicates a statistically significant difference using the Student's t-test, $p < 0.05$. C) Rat testicle of the control group. Leydig cells between seminiferous tubules (L). D) Rat testicle of DON group. Reduced number of Leydig cells between

seminiferous tubules (L). E) Seminiferous tubules of control animals. Sertoli cells between germ cells (arrow). F) Seminiferous tubules of animals exposed to DON. Reduced number of Sertoli cells between germ cells (arrow). HE. 25 µm bar.

Immunostaining of caspase-3 was used to evaluate the apoptotic index in germinative cells. No significant differences were observed in the different stages of development of the seminiferous cycle in treated or untreated animals (Figure 4).

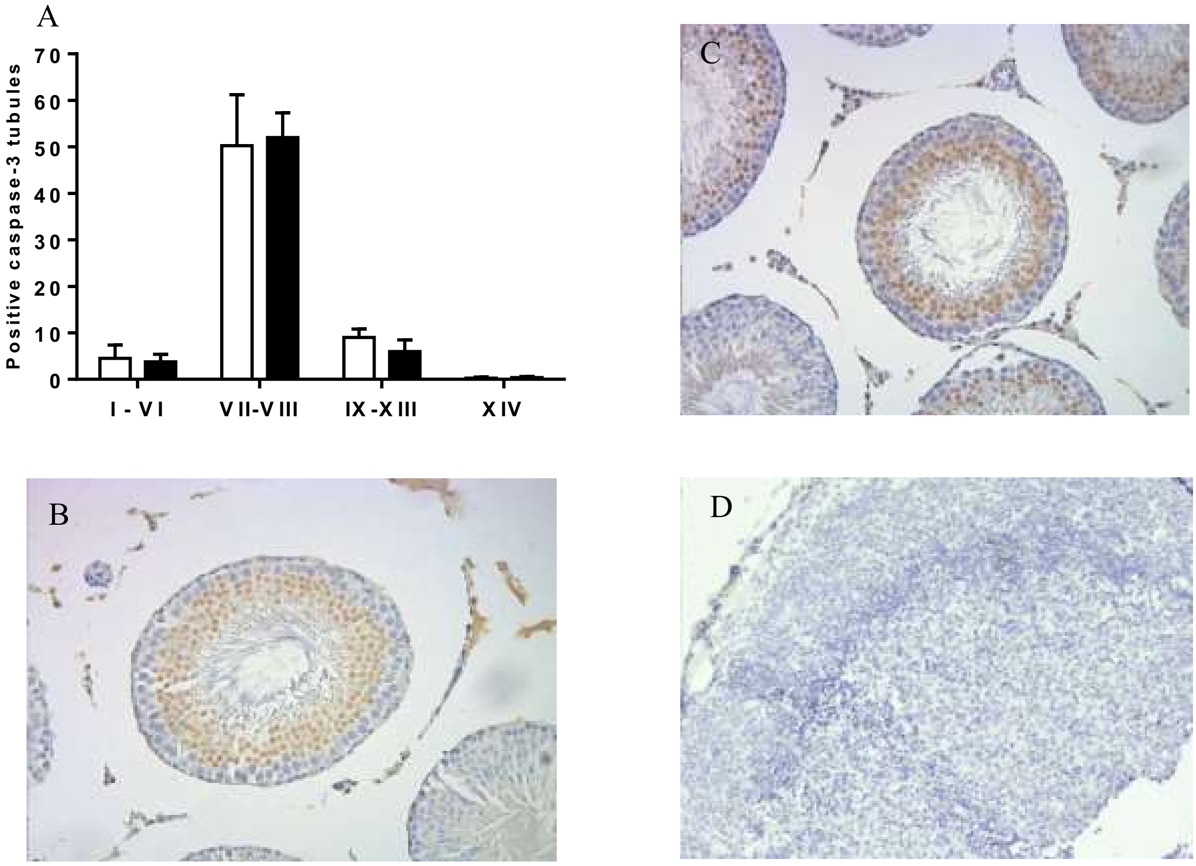

Figure 4. Effect of peripubertal exposure to deoxynivalenol (DON) on caspase-3 immunostaining in seminiferous tubules according to the different stages of the seminiferous epithelium cycle of rats. A) Number of seminiferous tubules stained for caspase-3 according to the kinetics of spermatogenesis. Experimental groups: (□) mycotoxin-free control diet or (■) contaminated with 9.4 mg/kg DON of feed from the postnatal day 28 to 56. Values are expressed as mean ± S.E.M. Student's t-test ($p \geq 0.05$). B) Seminiferous tubules of control

animals. Germ cells stained for caspase-3. C) Seminiferous tubules of animals exposed to DON. Germ cells stained for caspase-3. D) Lymph node section. Negative control. Lymphoid cells show no immunostaining for caspase-3. Immunoperoxidase. 75 µm bar.

Discussion and conclusion

The toxicity of DON in the immune and digestive systems is well established, however, the effects of this mycotoxin in the male reproductive system are little known. In addition, considering that children and adolescents showed a high risk of exposure to DON (Silva *et al.*, 2018; Papageorgiou et al., 2018), it is essential to identify and understand the potential reproductive effects of this mycotoxin at puberty. In this study, we have demonstrated that peripubertal exposure to DON can lead to growth retardation, changes in spermatogenesis, and testicular damage in the puberty period.

In animals and humans, the anorectic and emetic effects of DON have been evaluated (Maresca, 2013). In this study, a significant decrease in body weight gain and feed intake was observed in rats fed a DON-contaminated diet for 28 days during the peripubertal period. This finding is similar to previous studies, where the anorectic response to DON has been observed in adult rats and mice fed a diet contaminated with an analogous concentration of DON for a period of 30 and 17 days, respectively (Bracarense *et al.,* 2017; Clark *et al.,* 2015). In contrast, when male rats aging 21 days were exposed to a DON-contaminated diet containing 0.2 to 2 mg/kg DON for 42 days no effect was observed on feed intake, weight gain and anorexia (Rissato *et al.,* 2019). However, rodents, unlike humans, are better capable of metabolizing DON into DOM-1 (less toxic derivative) at the intestinal level (Lattanzio *et al.,* 2011), and this should be considered in interpreting these results. Given the major growth spurt that occurs at the time of puberty (Rogol *et al.*, 2002), the present results should be relevant for human consumer risk assessment.

The dynamics of spermatogenesis in the rat are composed of 14 cyclic stages. Each stage shows a determined period which is characterized by different generations of seminiferous epithelium cells since to have complete spermatogenesis it is essential that the germinative cell make 4.5 times the cycle of dynamics (Xiao *et al.,* 2014). Each stage has unique and fundamental processes to produce viable sperm. Thus, during stages I to VI, the spermatids are transported from the base of the apical compartment towards the luminal border. In stages VII to VIII occurs the spermiation and the transformation from type B spermatogonia into preleptotene spermatocyte. In stages IX to XIII, there is the differentiation of the preleptotene spermatocyte into the leptotene spermatocyte in which the cell moves towards the apical compartment according to the restructuring of the BHB. Finally, in stage XIV there is spermatocyte II meiosis (Clermont, 1972).

In the current study, the reduction in stage XIV revealed by the dynamics of spermatogenesis and the increase in the number of NORs during stages I-VI highlighted an acceleration in the differentiation of spermatocytes II for the phase of spermatids. This acceleration of stage XIV is also reported in Sprague-Dawley rats after 28 days of treatment with a dose of 5.0 mg/kg DON (Sprando *et al.,* 2005). In this study, it was also reported an early stretching of the elongated step 14 spermatids that occurs in stage XIV.

In prepubertal rats, it is expected to have a faster spermatogenic development than in sexually mature animals (Van Haaster and De Rooij, 1993). In the present study, we have detected a reduction in the time for sperm production and apparently, from spermatocyte II in the tubules XIV in animals treated with the contaminated diet. The understanding of this finding can be related to changes described in previous studies that show degeneration in spermatids that occur after stage XIV with the treatment of 5.0 mg/kg of DON in Sprague-Dawley rats (Sprando *et al.,* 2005).

Stage XIV consists of type A spermatogonia, pachytene spermatocytes in metaphase of meiosis, secondary spermatocytes and step 14 elongated spermatids, however 28 days earlier, these cells were type A spermatocytes and zygotene spermatocytes. In the rat, type A spermatocytes are in the basal compartment and do not express the P-glycoprotein (efflux pump highly expressed in the testicle). That is, as they are located outside BHB and do not express the efflux pump that expels and prevents the entry of xenobiotics, these cells become susceptible to the effects of substances such as DON (Su *et al.*, 2011; Xiao *et al.,* 2014). Therefore, this mycotoxin could be able to enter the seminiferous tubules indirectly using spermatocytes located outside BHB (Sprando *et al.,* 2005).

In the present study, consistently no changes were observed in the testicular weight (data not shown), tubular morphometry, and apoptotic index. Similar results were described in 28-day- old mice fed a DON-contaminated diet (10 mg/kg) for 90 days (Sprando *et al.,* 1999). Similar results were described in 28-day-old mice fed a DON-contaminated diet (10 mg/kg) for 90 days (Sprando *et al.,* 1999). On the contrary, recently a significant increase in the expression of apoptotic genes, such as Bim, caspase-3, caspase-8, and caspase- 9 were reported in the testicles of 42 days old mice receiving DON (2.4 mg/kg of body weight) for 28 days (Yang *et al.,* 2019). These contradictory data may be related to differences in experimental conditions used in each study. Factors as the animal species, dose, and age must be considered. All animal species evaluated to date are susceptible to the deleterious effects of DON, however, mice have been considered more susceptible when compared to rats (Eriksen and Pettersson, 2004). In addition, a possible approach to elucidate this issue would be to study cell death mechanisms (apoptosis, necrosis, and autophagy) induced by DON in testicles covering different types of cells of this organ.

Interestingly, DON-contaminated diet significantly reduced the number of Leydig cells (15.67%), which are responsible for hormonal production of testosterone (Zirkin and Papadopoulos, 2018). Accordingly, previous studies have shown that DON, among several mycotoxins, exhibits one of the greatest cytotoxicities for the MA-10 Leydig cells; and it was observed that doses from 0.1 µM of DON cause a significant reduction in cell viability in this cell line (Savard *et al.,* 2016). In addition, a decrease in progesterone production and the expression of the stAR and Nr4a1 proteins (precursor hormones of testosterone and essential proteins for steroidogenesis, respectively) was reported (Savard *et al.,* 2016). In accordance, a reduction in testosterone level has been reported after 28 days of treatment in Sprague-Dawley rats receiving a 5.0 mg/kg dose of DON (Sprando *et al.,* 2005). There was also a decrease in serum testosterone and the essential enzymes (Cyp11a1, Hsd3b1, Cyp17a1, 17-beta, and Hsd17b1) for its biosynthesis in mice receiving DON (1.2 mg/kg; 2.4 mg/kg and 4.8

mg/kg) for 28 days (Cao *et al.,* 2020). The decrease in Leydig cells causes a reduction in testosterone concentration, delaying and altering spermatogenesis and spermiogenesis, and consequently affecting pubertal development (Richmond and Rogol, 2007). Changes in Leydig cells have also the potential to disrupt the maintenance of BHB (Mruk and Cheng, 2015).

Sertoli cells contribute to spermatogenesis, acting as structural and nutritional support of germline cells. In this study, we noted that ingestion of DON induced a reduction in the number of Sertoli cells (8.76%), which can result in a lower daily production of sperm (Walker and Cheng, 2005). Similar results were observed by Sprando *et al.* (2005), where adult rats exposed to DON for 28 days showed intercellular vacuoles between Sertoli cells and germinative cells, and failures in the spermiation. In contrast, when mice (4 weeks old) were treated with a DON- contaminated diet (10 mg/kg of feed) for 90 days no changes in the amount of Sertoli cells were observed (Sprando *et al.*, 1999). The different results may be a result of species differences.

In addition, Sertoli cells are connected by specialized junction complexes providing the formation of BHB (Cheng and Mruk, 2002; 2012), thus, a reduction in the number of these cells could affect the maintenance of this exclusive and immune-privileged microenvironment, and consequently, DON may enter the seminiferous tubules (Franca *et al.*, 2005). As observed recently by Cao *et al.* (2020), the exposure of mice (5 weeks old) to DON for 28 days (2.4 mg/kg and 4.8 mg/kg) was responsible for causing a reduction in junction proteins (occludin, connexin 43, and N-cadherin) and damage to the integrity of BHB.

Furthermore, as the animals were exposed to mycotoxin after the 28th day of life, it can be said that the reduction in the number of Sertoli cells is closely related to their death, and not to lessened proliferation since these cells proliferate only until the 17th day after delivery in the rats (Xiao *et al.,* 2014).

Taken together, our results indicate that DON can alter sperm production, entering the seminiferous tubules and acting on germline cells probably in two ways: 1. by altering BHB (since there is a reduction in the number of Sertoli and Leydig cells, respectively responsible for its formation and maintenance), and 2. affecting the spermatocytes located in the basal compartment. Thus, the dynamics of spermatogenesis are altered as noticed by the acceleration of seminiferous stages. This change in dynamics can also be explained by the deficit in1 structural and nutritional support and by the reduction of Leydig cells that produce testosterone.

A high risk of exposure to mycotoxins in children and adolescents is a reality; however, the reproductive toxicity of these substances at puberty has been poorly assessed. This is the first study to evaluate the potential effects of peripubertal exposure to DON on the male reproductive system of pubertal animals. In conclusion, our results suggest a direct effect of DON on the dynamics of spermatogenesis, probably by decreasing the number of Sertoli and Leydig cells in pubertal animals. In this context, future studies on the association of DON and male fertility and puberty disorders are strongly suggested. Additionally, considering that after puberty a limited proliferative capacity is observed in these cell types (Tarulli *et al.*, 2012; Teerds and Huhtaniemi, 2015) possible persistent effects of DON on fertility and other reproductive parameters in adulthood also must be considered.

References

Bennett, J.W. and Klich, M, 2003. Mycotoxins. Clinical Microbiology Reviews 16: 497-516. https://doi.org/10.1128/CMR.16.3.497-516.2003

Bracarense A.P.F., Basso K.M., Da Silva E.O., Payros D. and Oswald I.P., 2017. Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. World Mycotoxin Journal 10: 89-96. https://doi.org/10.3920/WMJ2016.2094

Cao, Z., Huang, W., Sun, Y. and Li, Y., 2020. Deoxynivalenol induced spermatogenesis disorder by blood-testis barrier disruption associated with testosterone deficiency and inflammation in mice. Environmental Pollution 264: 114748. https://doi.org/10.1016/j.envpol.2020.114748

Chacur, M.G.M., Ibrahim, D.B., Arrebola, T.A.H., Sanches, O.C., Giuffrida, R., Oba, E. and Ramos, A.A, 2015. Avaliação da técnica de coloração AgNOR em testículos de ovinos. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 67: 447-454. https://doi.org/10.1590/1678-6695

Cheng, C.Y. and Mruk, D., 2002. Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiological Reviews 82: 825-874. https://doi.org/10.1152/physrev.00009.2002.

Cheng, C.Y. and Mruk, D., 2012. The blood-testis barrier and its implications for male contraception. Pharmacological Reviews 64: 16-64. https://doi.org/10.1124/pr.110.002790

Clark, E.S., Flannery, B.M. and Pestka, J.J., 2015. Murine anorectic response to deoxynivalenol (vomitoxin) is sex-dependent. Toxins (Basel) 7: 2845-2859. https://doi.org/10.3390/toxins7082845

Clermont, Y., 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiological Reviews 52: 198-236. https://doi.org/10.1152/physrev.1972.52.1.198.

Diamanti-Kandarakis, E. and Gore, A.C., 2011. Endocrine disruptors and puberty. Springer Science & Business Media, New York, USA. https://doi.org/10.1007-978-1-60761-561-3

Eriksen G.S. and Pettersson H., 2004. Toxicological evaluation of trichothecenes in animal feed. Animal Feed Science Technology 114:205–39. https://doi.org/10.1016/j.anifeedsci.2003.08.008

Eze, U.A., Huntriss, J., Routledge, M.N. and Gong, Y.Y., 2018. Toxicological effects of regulated mycotoxins and persistent organochloride pesticides: In vitro cytotoxic assessment of single and defined mixtures on MA-10 murine Leydig cell line. Toxicology in Vitro 48: 93-103. https://doi.org/10.1016/j.tiv.2017.12.019

Favareto, A.P., Fernandez, C.D., Da Silva, D.A., Anselmo-Franci, J.A. and Kempinas, W.G., 2011. Persistent impairment of testicular histology and sperm motility in adult rats treated with Cisplatin at peri-puberty. Basic and Clinical Pharmacology and Toxicology 109: 85-96. https://doi.org/10.1111/j.1742-7843.2011.00688.x

Franca, L.R., Avelar, G.F. and Almeida, F.F., 2005. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. Theriogenology 63: 300-318. https://doi.org/10.1016/j.theriogenology.2004.09.014

Gonçalves, G.D., Vieira, N.A., Vieira, H.R., Valerio, A.D., Siervo, G.E.M.L., Pinheiro, P.F.F., Martinez, F.E., Guarnier, F.A., Teixeira, G.R. and Fernandes, G.S.A., 2017. Role of resistance physical exercise in preventing testicular damage caused by chronic ethanol consumption in UChB rats. Microscopy Research and Technique 80: 378-386. https://doi.org/10.1002/jemt.22806

Grenier B. and Applegate T.J., 2013. Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals. Toxins (Basel) 5: 396-430. https://doi.org/10.3390/toxins5020396

Guerra, M.T., Sanabria, M., Leite, G.A., Borges, C.S., Cucielo, M.S., Anselmo-Franci, J.A., Foster, W.G. and Kempinas, W.G., 2017. Maternal exposure to butyl paraben impairs testicular structure and sperm quality on male rats. Environmental Toxicology 32: 1273-1289. 44 https://doi.org/10.1002/tox.22323

Khoury, D.E., Fayjaloun, S., Nassar, M., Sahakian, J. and Aad, P.Y., 2019. Updates on the effect of mycotoxins on male reproductive efficiency in mammals. Toxins 11: 515. https://doi.org/10.3390/toxins11090515

Lattanzio V.M.T., Solfrizzo, M., De Girolamo, A., Chulze, S.N., Torres, A.M. and Visconti, A., 2011. LC-MS/MS characterization of the urinary excretion profile of the mycotoxin deoxynivalenol in human and rat. Journal of Chromatography B 879: 707–715. https://doi.org/10.1016/j.jchromb.2011.01.029

Leblond, C.P. and Clermont, Y., 1952. Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfurous acid technique. American Journal of Anatomy 90: 167-215. https://doi.org/10.1002/aja.1000900202

Lee, H.J. and Ryu, D., 2017. Worldwide occurrence of mycotoxins in cereals and cereal-derived food products: public health perspectives of their co-occurrence. Journal of Agricultural and Food Chemistry 65: 7034-7051. https://doi.org/10.1021/acs.jafc.6b04847

Maresca, M., 2013. From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. Toxins 5: 784-820. https://doi.org/10.3390/toxins5040784

McCormick, S.P., Stanley, A.M., Stover, N.A. and Alexander, N.J., 2011. Trichothecenes: from simple to complex mycotoxins. Toxin (Basel) 3: 802 – 814. https://doi.org/10.3390/toxins3070802

Mishra, S., Dixit, S., Dwivedi, P.D., Pandey, H.P. and Das, M., 2014. Influence of temperature and pH on the degradation of deoxynivalenol (DON) in aqueous medium: comparative cytotoxicity of DON and degraded product. Food Additives & Contaminants: Part A. Chemistry, Analysis, Control, Exposure & Risk Assessment 31: 121-131. https://doi.org/10.1080/19440049.2013.861613

Mruk, D.D. and Cheng, C.Y., 2015. The mammalian blood-testis Barrier: its biology and regulation. Endocrine Reviews 36: 564-591. https://doi.org/10.1210/er.2014-1101

Nassr, A.C., Arena, A.C., Toledo, F.C., Bissacot, D.Z., Fernandez, C.D., Spinardi-Barbisan, A.L., Pires, P.W. and Kempinas, W.G., 2010. Effects of gestational and lactational fenvalerate exposure on immune and reproductive systems of male rats. Journal of Toxicology and Environmental Health. Part A 73: 952-964. https://doi.org/10.1080/15287391003751745

Ojeda, S.R., Andrews, W.W., Advis, J.P. and White, S.S., 1980. Recent advances in the endocrinology of puberty. Endocrine reviews 1: 228-257. https://doi.org/10.1210/edrv-1-3-228

Papageorgiou, M., Wells, L., Williams, C., White, K., De Santis, B., Liu, Y., Debegnach, F., Miano, B., Moretti, G., Greetham, S., Brera, C., Atkin, S.L., Hardie, L.J. and Sathyapalan, T., 43 2018. Assessment of urinary deoxynivalenol biomarkers in UK children and adolescents. Toxins 10: 50. https://doi.org/10.3390/toxins10020050

Pestka, J., 2007. Deoxynivalenol: toxicity, mechanisms, and animal health risks. Animal Feed Science and Technology 137: 283-298. https://doi.org/10.1016/j.anifeedsci.2007.06.006

Pestka, J.J. and Smolinski, A.T., 2005. Deoxynivalenol: toxicology and potential effects on humans. Journal of Toxicology and Environmental Health. Part B 8: 39-69. https://doi.org/10.1080/10937400590889458

Pestka, J.J., Uzarski, R.L. and Islam, Z., 2005. Induction of apoptosis and cytokine production in the Jurkat human T cells by deoxynivalenol: role of mitogen-activated protein kinases and comparison to other 8-ketotrichothecenes. Toxicology 206: 207-219. https://doi.org/10.1016/j.tox.2004.08.020

Richmond, E.J. and Rogol, A.D., 2007. Male pubertal development and the role of androgen therapy. Nature Clinical Practice Endocrinology & Metabolism 3: 338-344. https://doi.org/10.1038/ncpendmet0450

Rissato D.F., de Santi Rampazzo A.P., Borges S.C., Sousa F.C., Busso C., Buttow N.C. and Natali M.R.M., 2020. Chronic ingestion of deoxynivalenol-contaminated diet dose-dependently decreases the area of myenteric neurons and gliocytes of rats. Neurogastroenterology & Motility 32: e13770. https://doi.org/10.1111/nmo.13770

Rogol A.D., Roemmich, J.N. and Clark, P.A.; 2002. Growth at puberty. Journal of Adolescent Health 31: 192-200. https://doi.org/10.1016/S1054-139X(02)00485-8

Savard, C., Nogues, P., Boyer, A. and Chorfi, Y., 2016. Prevention of deoxynivalenol- and zearalenone-associated oxidative stress does not restore MA-10 Leydig cell functions. Toxicology 341: 17-27. https://doi.org/10.1016/j.tox.2016.01.003

Siervo, G.E.M.L., Vieira, H.R., Ogo, FM, Fernandez, C.D.B., Gonçalves, G.D., Mesquita S.F.P., Anselmo-Franci, J.Ap., Cecchini, R., Guarnier F.A. and Fernandes, G.S.A., 2015. Spermatic and testicular damages in rats exposed to ethanol: influence of lipid peroxidation but not testosterone. Toxicology 330: 1-8. https://doi.org/10.1016/j.tox.2015.01.016

Silva, M.V., Pante, G.C., Romoli, J.C.Z., de Souza, A.P.M., da Rocha, G.H.O., Ferreira, F.D., Feijó, A.L.R., Moscardi, S.M.P., de Paula, K.R., Bando, E., Nerilo S.B. and Machinski Jr, M., 2018. Occurrence and risk assessment of population exposed to deoxynivalenol in foods derived from wheat flour in Brazil. Food Additives & Contaminants 35: 546-554. https://doi.org/10.1080/19440049.2017.1411613

Soler, L. and Oswald, I.P., 2018. The importance of accounting for sex in the search of proteomic signatures of mycotoxin exposure. Journal of Proteomics 178: 114-122. https://doi.org/10.1016/j.jprot.2017.12.017

Sprando, R.L., Collins, T.F., Black, T.N., Olejnik, N., Rorie, J.I., Eppley, R.M. and Ruggles, D.I., 2005. Characterization of the effect of deoxynivalenol on selected male reproductive endpoints. Food and Chemical Toxicology 43: 623 – 635. https://doi.org/10.1016/j.fct.2004.12.017

Sprando, R.L., Pestka, J., Collins, T.F., Rorie, J., O'Donnell, M., Hinton, D. and Chirtel, S., 1999. The effect of vomitoxin (Deoxnivalenol) on testicular morphology, testicular spermatid counts and epididymal sperm counts in IL-6KO [B6129-IL6 [TmlKopf] (IL-6 gene deficient)] and WT [B6129F2 (wild type to B6129-IL6 with an intact IL-6 gene)] mice. Food and Chemical Toxicology 37: 1073-1079. https://doi.org/10.1016/S0278-6915(99)00103-9

Su, L., Mruk, D.D. and Cheng, C.Y., 2011. Drug transporters, the blood–testis barrier, and spermatogenesis. The Journal of Endocrinology 208: 207-223. https://doi.org/10.1677/JOE-10-0363

Tarulli G.A., Stanton P.G. and Meachem S.J., 2012. Is the adult sertoli cell terminally differentiated?. Biology of Reproduction 87: 13, 1-11. https://doi.org/10.1095/biolreprod.111.095091

Teerds K.J., and Huhtaniemi I.P., 2015. Morphological and functional maturation of leydig cells: from rodent models to primates. Human Reproduction Update 21: 310–328. https://doi.org/10.1093/humupd/dmv008

Urbanek, K.A., Habrowska-Gorczynska, D.E., Kowalska, K., Stanczyk, A., Domińska, K. and Piastowska-Ciesielska, A.W., 2018. Deoxynivalenol as potential modulator of human steroidogenesis. Journal of Applied Toxicology 38: 1450-1459. https://doi.org/10.1002/jat.3623

Van Haaster, L.H. and De Rooij, D.G., 1993. Spermatogenesis is accelerated in the immature djungarian and chinese hamster and rat. Biology of Reproduction 49: 1229-1235. https://doi.org/10.1095/biolreprod49.6.1229

Walker, W.H. and Cheng, J., 2005. FSH and testosterone signaling in Sertoli cells. Reproduction 130: 15-28. https://doi.org/10.1530/rep.1.00358

Xiao, X., Mruk, D.D., Wong, C.K. and Cheng, C.Y., 2014. Germ cell transport across the seminiferous epithelium during spermatogenesis. Physiology (Bethesda) 29: 286-298. https://doi.org/10.1152/physiol.00001.2014.

Yang, J.H., Wang, J.H., Guo, W.B., Ling, A.R., Luo, A.Q., Liu, D., Yang, X.L. and Zhao, Z.H., 2019. Toxic effects and possible mechanisms of deoxynivalenol exposure on sperm and testicular damage in balb/c mice. Journal of Agricultural and Food Chemistry 67: 2289-2295. https://doi.org/10.1021/acs.jafc.8b04783

Zirkin, B.R. and Papadopoulos, V., 2018. Leydig cells: formation, function, and regulation. Biology of Reproduction 99: 101-111. https://doi.org/10.1093/biolre/ioy059

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Effects of deoxynivalenol exposure at peripuberty over testicles of rats: structural and functional alterations

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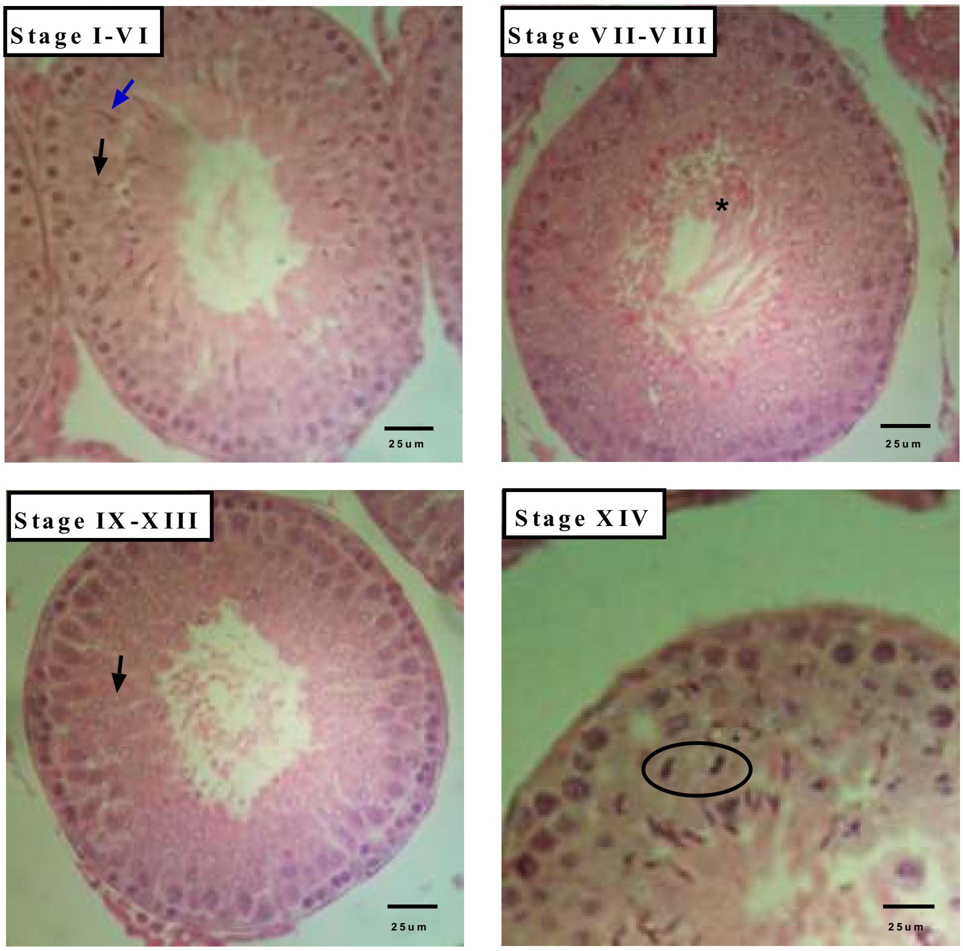

Black arrows represent rounded cells, while blue arrows signal elongated spermatids. Seminiferous tubules that have both generations of spermatids (rounded and elongated) are classified in stages I-VI. Tubules that have only one generation are classified in stages IX-XIII. Asteriks signals sperm in the tubule lumen, which are classified into stages VII-VIII. Stage XIV is characterized by the presence of cell in cell division, as represented by the circle.

4.3 Exposure to deoxynivalenol during peripubertal periods induces ovarian damage in immature rats

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Abstract

Puberty is a process regulated by the endocrine system in which several physiological and biological changes are responsible for the beginning of the reproductive functions in animals and humans. DON can interfere with the hormonal system, thereby inducing endocrine disorders and reproductive damage. However, the potential of juvenile and peripubertal exposure to DON on ovaries has been overlooked. Considering that children and adolescents show a high-risk exposure to DON, the present study evaluated the effect of this mycotoxin during puberty on follicular integrity and the expression of pro-apoptotic proteins (BAX and Caspase-3) and anti-apoptotic protein (BCL-2) in the ovarian tissue of young rats. Ten juvenile Wistar rats (28 days old) were used. Two groups were exposed to the following experimental diets for 28 days: 1) control: mycotoxin-free diet and 2) DON: a diet containing 10 mg DON/Kg of feed. In the end, animals (56 days old) were euthanized, and the ovaries and uterus were weighted. Ovaries were destined for morphometrical and immunohistochemical evaluation. After exposure to DON, a significant reduction was observed in the weight of the ovaries and uterus. Ingestion of DON induced no changes in the number of healthy ovarian follicles or atretic follicles at all developmental stages. A significant increase in the expression of BAX and Caspase-3 and a decrease in the expression of BCL-2 were observed in the majority of follicular stages and corpora lutea in the ovaries exposed to DON. In conclusion, exposure to DON during the juvenile and peripubertal periods can interfere with the apoptotic pathways in different populations of ovarian cells in the early life of adult animals.

Keywords: Deoxynivalenol, ovary, follicular integrity, apoptosis, rat.

1. Introduction

Puberty is the period of transition between childhood and adulthood characterized by the development of secondary sexual characters, acceleration of growth, and the beginning of reproductive functions (Maranghi and Mantovani, 2012). In rats, the transition from the juvenile or prepubertal stage (~21 days of age; no fluid in the uteri) to the peripubertal stage (~32 days of age, fluid in the uteri) is critical for the achievement of puberty (Ojeda et al, 1980). The first estrous cycle is an event that marks the end of a complex cascade of hormonal change that occurs around the pos natal day (PND) 34 to 38 (Ojeda et al, 1980). Initially, this cycle is irregular reaching stability and maturity approximately on the PND 65 (AGARWAL *et al.*, 2012). Therefore, the peripubertal period of the ovaries is a large window of vulnerability to the action of toxic agents. Deoxynivalenol or DON, a mycotoxin produced by *Fusarium* species is frequently detected in staple food crops like wheat, maize, rye, barley, oats, and other cereals throughout the world, thus making it a potential health hazard (Mishra et al., 2020). Interestingly, in Brazil considering the consumption of bread and pasta, females of all age groups (teenagers, adults, and elderly) are exposed to DON at higher levels when compared to males (Silva et al., 2018). In addition, according to recent studies, DON has been found at higher levels in the urine of children and adolescents compared to adults and elderly, indicating an increased risk of exposure to DON, especially for this population (De Santis et al., 2019; Deng et al., 2018; Silva et al., 2018). However, the potential of this mycotoxin in acting over the biological events that occur during female juvenile and peripubertal period has been neglected so far.

In animals, exposure to high doses of DON causes diarrhea, vomiting, leukocytosis, and hemorrhage, while chronic exposure to low doses has been associated with anorexia, reduced weight gain, and feeding efficiency as well as changes in the immune, neuroendocrine system, and reproductive system (Knutsen et al., 2017). In *in vitro* models, a direct effect of DON on porcine oocytes and granulosa cells has been observed (Yu et al., 2017). DON affects porcine oocytes maturation, inducing nuclear malformations, and disrupting meiotic spindle, oxidative stress, autophagy, apoptosis, and epigenetic modifications (Alm et al., 2002; Han et al., 2016; Lan et al., 2018; Malekinejad et al., 2007; Schoevers et al., 2010). Moreover, alterations in cell proliferation, steroidogenesis, apoptosis, and genetic changes in important metabolic pathways have been identified in DON-exposed porcine granulosa cells (Medvedova et al., 2011; Ranzenigo et al., 2008; Yang et al., 2020).

In *in vivo* studies using adult rodents, a diet with 20 mg DON/kg of feed provided to males and females for 60 and 15 days, respectively, resulted in a significant reduction in the fertility rate compared to the control group, since only 50% of mating in the treated group resulted in pregnancy compared to 80% in the control group (Morrissey and Vesonder, 1985). More specifically, ovaries of adult rats exposed to DON showed a decrease in the expression of growth factor similar to insulin I and the antiapoptotic protein BCL-2 associated with an increase in the levels of pro-apoptotic proteins BAX and Caspase-3 (Kolesárová et al., 2012). After seven days of DON intake, female mice showed oocytes with significant alterations in the maturation rate, meiotic spindle formation, and oxygen reactive species level resulting in lower fertilization rates. Thereby, few *in vivo* studies have evaluated the direct ovarian effect of DON (Yu et al., 2017), and no reports have analyzed the action of this toxin on the reproductive function development at puberty.

Taking into account the factors mentioned above, the present study aimed to evaluate whether exposure to a DON-contaminated diet during juvenile and periubertal period can cause adverse effects on follicular integrity in the ovaries of young Wistar rats, as well as to verify the involvement of apoptotic signaling pathways on DON-related follicular toxicity.

2. Materials and Methods

2.1. Animals and diet

Ten female Wistar rats, 21-days-old, were carefully kept under pathogen-free conditions and housed in adequate conditions for seven days before the beginning of the experiments. The animals were fed a standard diet (Quimtia®) and water *ad libitum*. During the experiment, the animals were kept in a room with adequate temperature for thermal comfort (± 21°C), adequate ventilation (exhaustion of air), humidity (40-70%), and photoperiod (12h of light and 12h of dark). The project was previously presented and approved by the Animal Use Ethics Committee of our institution (CEUA protocol nº 6986.2017.27).

2.2. Experimental design

Juvenile animals (n = 10) with 28 days postnatal day (Ojeda et al. 1980) were randomly assigned to two experimental groups: Control group (n = 5, mycotoxin-free diet) and DON group (n = 5, diet containing 10 mg DON/kg of food). A crude extract of DON (40 ppm) produced at the Laboratory of Mycology, Luiz de Queiroz College of Agriculture, Universidade de São Paulo, was used in the preparation of the contaminated diet. A blend (standard diet+DON) was prepared at Universidade Estadual de Londrina facilities using a commercial feed mixer. The diets were sent to the Samitec laboratory (Santa Maria – RS/Brazil), where the mycotoxins levels were assessed using the HPLC/MS-MS method. The control diet showed lower levels of DON than the limit of quantification established (200 µg/kg with a stated accuracy of 101%). According to Grenier and Applegate (2013), the dose of DON used in the treatment (10 mg/kg of feed) corresponds to a dose occasionally found in commodities, feeds, and feed ingredients. However, a realistic dose was not chosen since a higher metabolism of DON to DOM-1 is found in rats when compared to humans (Lattanzio et al., 2011). In addition, the chosen dose is in agreement with other relevant studies that have evaluated the toxicity of DON in the murine species (Bracarense et al., 2017; Clark et al., 2015). The animals were fed a control or a contaminated diet for 28 days. Thus, after the experimental period, euthanasia was performed using an intraperitoneal overdose of sodium pentobarbital, which was confirmed by dislocation of the cervical segment of the spinal cord and decapitation.

2.3. Zootechnical parameters and weight of the ovary and uterus

During the experiment, the animals were weighed weekly and body weight gain and food intake were calculated for each week. In addition, after euthanasia, the ovary and uterus were carefully collected and weighed.

2.4. Follicular integrity

The ovaries were collected, fixed in 10% buffered formalin solution, and submitted to routine histological processing. Histological sections were stained with hematoxylin-eosin. For the counting of ovarian follicles and corpus luteum, samples were subjected to optical microscopy, analyzing one section (4-µm-thick), and neglecting the nine subsequent sections, up to the hundredth histological section. The follicles were divided into three categories according to the classification by Pedersen and Peters, 1968): (1) primordial and primary follicles (characterized by oocytes surrounded by a single layer of flattened and cubic granulosa cells, respectively); (2) growing follicles (consisting of more than one layer of granulosa cells) and preantral (more than one layer of granulosa cells with outline of follicular antrum); (3) antral follicles (presence of a well-developed follicular antrum) and mature follicles (with the oocyte displaced to the periphery of the antrum surrounded by cells of the radial crown). In addition, each ovarian follicle was classified as healthy or atretic. Thus, in each section, the total number of each follicular type and corpus luteum was observed, and after the analysis of 10 sections per animal, an average number of each follicular type and corpus luteum was obtained per animal.

2.5. Immunohistochemical analyses

Programmed cell death biomarkers were evaluated using the immunohistochemical assay. This analysis was performed using primary antibodies (BAX and Caspase-3 as apoptosis inducers) and BCL-2 (an apoptosis inhibitor), according to the protocol outlined by Chuffa et al. (2016). For the immunohistochemical procedures, the slides with ovarian tissue were deparaffinized in xylene, and slides were immersed in 0.01M sodium citrate buffer and subjected to the microwave (700-800W) at pH 6.0 for antigen retrieval. After blocking the endogenous peroxidase activity, the tissue sections were incubated with BSA (3%) for a period of 1 h to avoid nonspecific binding. Afterwards, the slides were incubated overnight in a humidified chamber at 4°C with the primary antibodies (Abcam, Cambridge, UK): rabbit polyclonal anti-BCL-2 (1: 100), polyclonal anti-BAX rabbit, and rabbit monoclonal anti-caspase-3 (1:50). Then, TBS-T buffer was used to wash these slides and then incubated with the secondary antibody (anti-mouse IgG or anti-rabbit IgG polymer; DakoCytomation, Carpinteria, CA, USA) at room temperature for 1 h. Subsequently, these sections reacted with diaminobenzidine (Sigma – Aldrich) for 5 minutes. Finally, they were contrasted using hematoxylin staining. For the ovarian structures, all follicular and luteal cells present in the ovarian follicles and the corpus luteum were counted. Using the same criteria, the counting of cells positive for BCL-2, BAX, and caspase-3 was performed. Through the number of follicular or luteal cells positive for BCL-2, BAX, and Caspase-3 by the total cell number in each ovarian follicle or corpus luteum, the average percentage (%) of immunostaining for each protein present in ovarian follicles and corpus luteum was calculated. Regarding the stromal cell and oocyte immunoreactions, the analysis was performed based on staining intensity that was scored as absent, weak, moderate, or strong. Whenever possible, 10 fields were used for each section.

2.5 Statistical analysis

The data were expressed as mean ± standard deviation. After verifying the assumptions of normality of residues (Shapiro's test) and homogeneity of variances (Bartlett's test), the data were analyzed by Student's t test. Statistical significance was set at $P < 0.05$.

3. RESULTS

3.1. DON affects the weight of reproductive organs

As observed in Figure 1, there is no statistical difference in body weight, gain weight, and feed intake between the experimental groups. However, the exposure to a DON-contaminated diet decreased the weight of the ovaries and uterus by almost half (43.23%) (Figure 1D).

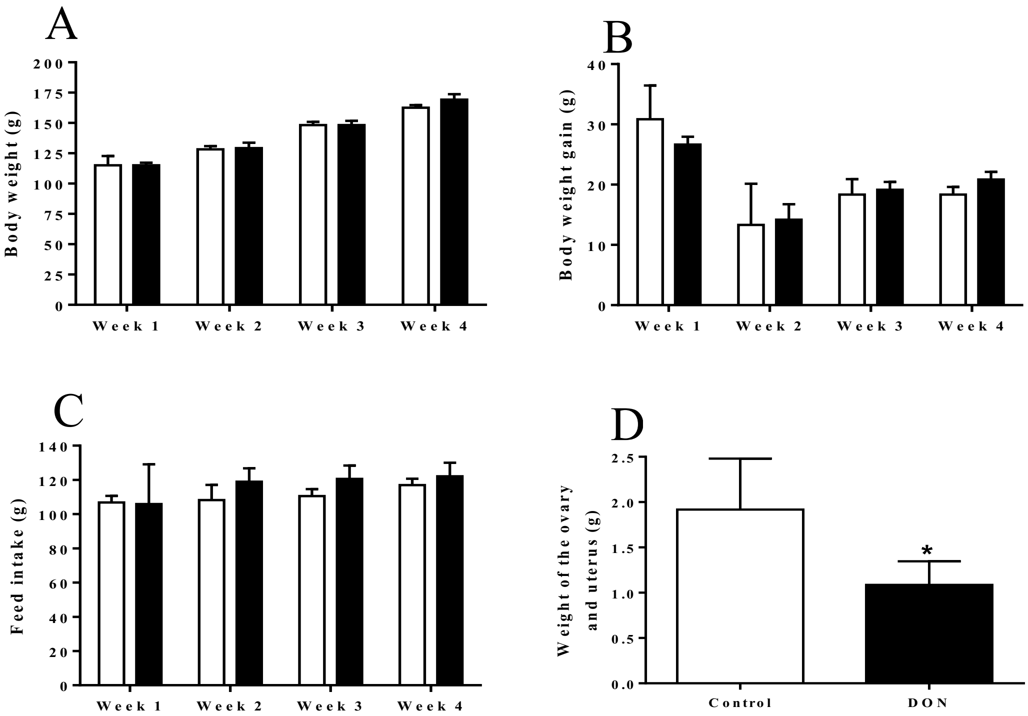

Figure 1. Effect of deoxynivalenol (DON) exposure on zootechnical parameters and weight of ovaries and uterus of young rats. A) Weekly body weight (g - grams), B) weekly body weight gain (g), C) weekly feed intake (g), and D) weight of the animal's ovary and uterus (g). Data are presented as mean ± standard deviation (n = 5/group). * $P < 0.05$ indicates statistical significance. Student's t-test. Control group (□) and DON-treated group (■).

3.2 DON induced no change in follicular counting and integrity when consumed during peripubertal periods

After exposure to DON no change was observed in the numbers of healthy and atretic ovarian follicles according to the stages of follicular development in the ovaries of rats exposed to a diet DON-contaminated (Table 1).

Table 1. Numbers of healthy and atretic ovarian follicles according to the stages of follicular development in the ovaries of rats exposed to a diet contaminated with deoxynivalenol (10 mg DON/kg of food) from the postnatal days 28 to 56.

	Primordial and primary follicles		Growing follicles		Pre Antral follicles		Antral follicles		Mature follicles		Total follicles	
	Healthy	Atretic	Healthy	Atretic	Healthy	Atretic	Healthy	Atretic	Healthy	Atretic	Healthy	Atretic
Control	15.57± 10.18	0.66± 0.10	3.86± 1.17	1.76± 1.53	2.15± 1.18	0.37± 0.25	1.44± 0.81	0.90± 0.79	0.36± 0.19	0.91± 0.51	23.63± 14.98	4.66± 3.35
DON	20.78± 10.74	0.11± 0.01	2.98± 0.36	0.28± 0.18	1.43± 0.81	0.12± 0.05	1.06± 0.49	0.41± 0.07	0.38± 0.24	0.59± 0.40	26.64± 12.67	1.52± 1.27

Data are presented as mean ± standard deviation (n = 5). Student’s t-test ($p \leq 0.05$).

3.2. Immunohistochemical analysis

Using immunohistochemical assays, the protein expressions of BCL-2 (anti-apoptotic), BAX, and Caspase-3 (pro-apoptotic) were evaluated in the ovaries of young rats in both groups (Figures 2 and 3). Regarding ovarian follicles, BCL-2 showed a significant reduction in the immunostaining of primordial and primary follicles ($p<0.001$), growing ($p<0.0001$), preantral ($p<0.001$), antral ($p=0.006$), and mature follicles ($p=0.002$), as well as in the corpus luteum ($p=0.02$) in animals exposed to DON when compared to the control group (Figure 2A). After treatment with DON, the ovarian stroma and the cytoplasm of the oocytes showed immunoreaction varying from weak to moderate, compared to the strong and diffuse immunostaining in the ovarian stroma of the control animals, demonstrating that the mycotoxin decreased the expression of this anti-apoptotic protein in the ovaries of young rats (Figure 3A-3D).

However, the number of BAX-positive cells was significantly higher in all stages of the ovarian follicles and corpus luteum in the DON-exposed group. There was a significant increase of 6.98% ($p=0.009$), 8.33% ($p=0.0009$), 11.95% ($p=0.0006$), 3.51% ($p=0.03$), 8.59% ($p=0.02$), 12.52% ($p=0.0006$) in the primordial and primary follicles, growing, preantral, antral, mature follicles, and corpus luteum, relatively to the control animals, respectively (Figure 2B). Considering other ovarian structures, weak immunostaining with the absence of BAX protein was observed in the control group (Figures 3E and 3F), whereas in the ovaries exposed to DON, a moderate expression was found in stromal cells and in the cytoplasm of oocytes of growing follicles (Figure 3G-3H).

Consequently, the DON-exposed group also demonstrated a significant increase in the number of Caspase-3 positive cells compared to the control group. This significant increase was 22.97% ($p<0.0001$), 1.42% ($p<0.0001$), 1.60% ($p=0.0007$), 51.61% (p<0, 0001) in the primordial and primary, growing, mature follicles, and in the corpus luteum of DON-exposed ovaries (Figure 2C). For stromal cells, the Caspase-3 immunostaining varied from absent to weak in the control group (Figure 3I, 3K and 3L), while a moderate to strong expression of Caspase-3 was observed in the ovarian stroma of animals of DON group (Figure 3J, 3M and 3N).

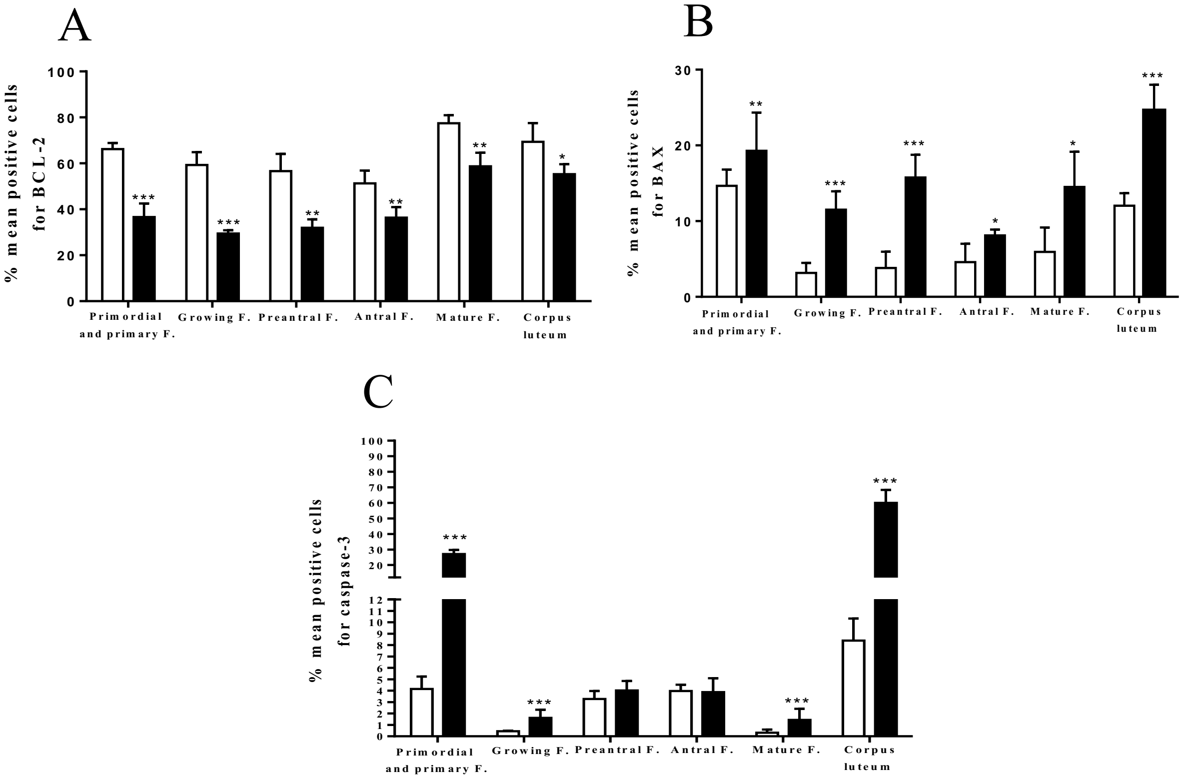

Figure 2. Effect of deoxynivalenol (DON) exposure on immune expression of BCL-2, BAX, and Caspase-3 proteins in the ovaries of young rats. Percentage of immune-positive cells for BCL-2 (**A**), BAX (**B**), and Caspase-3 (**C**) proteins in ovarian follicles (F.) at different developmental stages and corpus luteum in the ovaries of rats exposed to a control diet free of mycotoxins (□) or contaminated with 10mg/kg of DON (■) from postnatal day 28 to 56. Values are expressed as mean ± standard deviation. Student's t-test *** p <0.0001; ** p <0.001, * p <0.05.

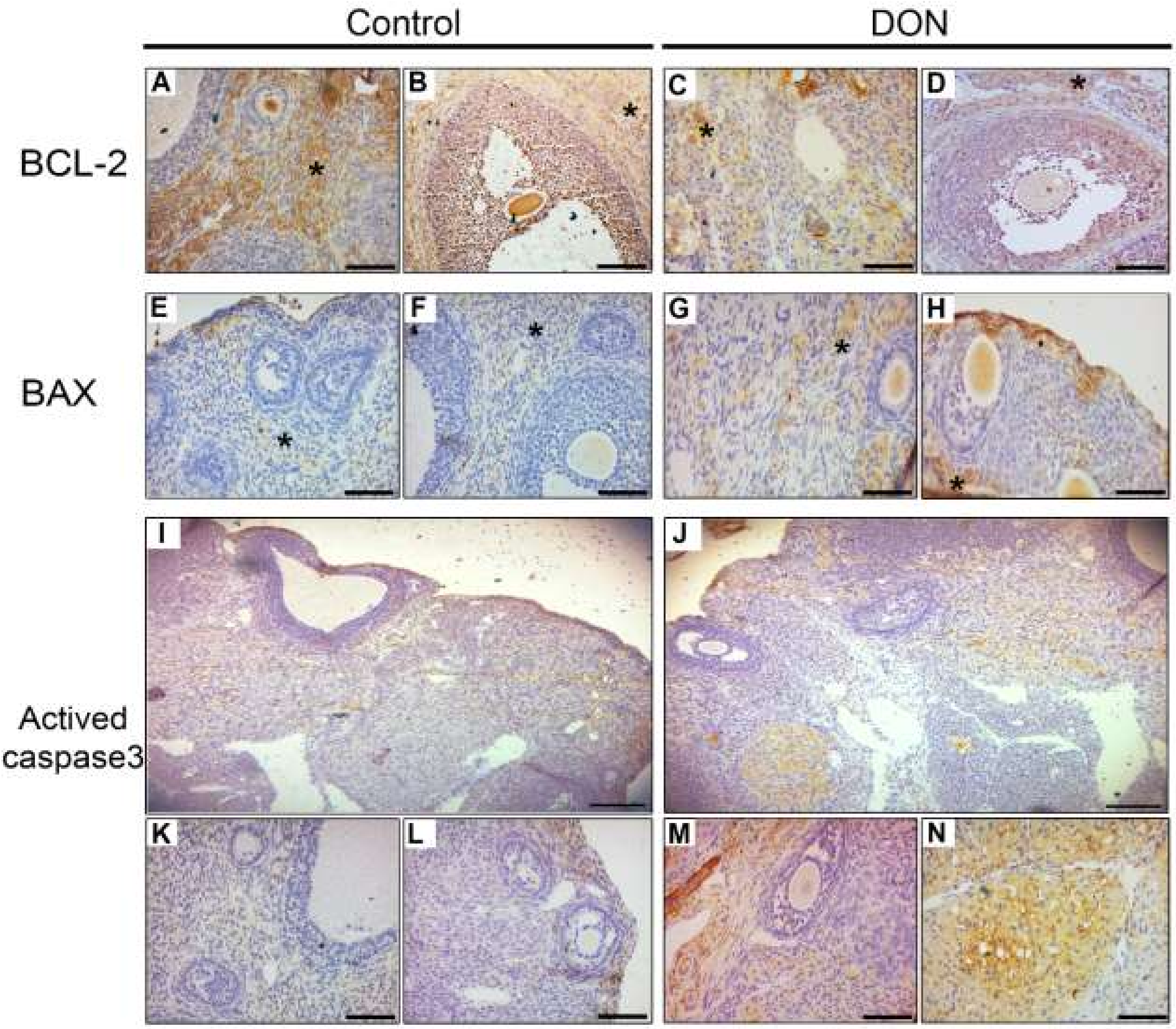

Figure 3. Effect of deoxynivalenol (DON) exposure on BCL-2, BAX and Caspase-3 apoptosis signaling pathways in the ovaries of rats exposed to a mycotoxin-free control diet and a diet contaminated with 10 mg DON/kg during the PND 28 to 56. (**A** and **B**) Ovaries of control animals showing strong BCL-2 immunostaining in the ovarian stroma (*), granulosa cells, and oocyte. (**C** and **D**) Ovaries of animals exposed to DON showing BCL-2 staining with less intensity in the ovarian stroma (*) compared to the control. (**E** and **F**) Control ovaries with weak immunostaining of BAX protein (*). (**G** and **H**) DON-treated animals showing moderate BAX staining in the ovarian stroma (*), oocytes, and few granulosa cells. (**I**, **K** and **L**) Control group with weak to absent Caspase-3 immunostaining in the ovarian stroma. (**J**, **M** and **N**) DON-treated animals showing moderate to strong immunoreaction for the Caspase-3 in the ovarian stroma and corpus luteum. For A-H and K-L images, scale bars = 50 µm; I-J images, scale bars = 100 µm.

4. Discussion

Puberty is a complex process of profound morphological, endocrine, and behavioral changes, through which an immature individual becomes a fertile adult. Over the past few decades, toxicological studies have identified the potential effects of specific substances on the development of reproductive functions (Maranghi and Mantovani, 2012). Among these studies, we highlight those involving the potential of mycotoxins to cause disturbances in puberty and impaired fertility in animals and humans (Eze et al., 2018; Massart et al., 2008; Massart and Saggese, 2010; Yang et al., 2015). However, the effects of exposure on peripubertal animals to DON are yet unknown. Considering further that higher urinary levels of DON are observed in children and adolescents compared to adults (De Santis et al., 2019; Deng et al., 2018; Silva et al., 2018), a better understanding on the reproductive toxicity of DON in pubescent animals may provide important information on possible damage of DON in puberty of humans.

Initially, the effect of DON was evaluated on the zootechnical parameters and the ovaries and uterus weight. In accordance with previous studies (Andretta et al., 2012; Clark et al., 2015; Iverson et al., 1995; Rotter et al., 1994), DON resulted in no effect on body weight, body weight gain, and feed intake of females. However, a significant reduction in the ovaries and uterus weight was observed in the animals exposed to the contaminated diet, indicating a possible toxic effect of DON on these organs in young females.

Concerning follicular integrity of pubescent ovaries, no change was observed. Similar results were observed in previous studies, where rats fed a DON-contaminated diet (0–2mg kg/bw/day) for 60 days before and during pregnancy showed no histological abnormalities in the ovaries of dams and pups (Morrissey, 1984; Morrissey and Vesonder, 1985). In addition, when pigs were exposed to sub chronic or chronic ingestion of DON-contaminated diets no significant difference in the histologic analysis of ovaries was observed (Friend et al., 1986; Trenholm et al., 1994). In contrast, an opposite result was found in our previous *ex vivo* studies, where swine ovarian explants exposed to 10 μM DON (equivalent to 3 mg/kg of feed) resulted in damage to follicular integrity characterized by a reduction in the number of normal ovarian follicles and an increase in degenerated follicles at all stages of follicular development (Gerez et al., 2021; Gerez et al., 2017). Thereby, these divergent results can probably be related to the different methods (*ex vivo* and *in vivo*) and exposure mode to toxin that is an important variable in assessing reproductive toxicity of DON.

During puberty, apoptosis in ovarian follicles represents a determinant process in reproductive longevity (Liew et al., 2017). In our study, the effects of DON on apoptotic pathways signaling were verified. DON resulted in a significant increase in the expression of apoptotic pathways (BAX and Caspase-3) associated with a decrease in anti-apoptotic expression (BCL-2). Ovarian changes induced by DON have been previously described and revealed similarities to those observed in this study. In ovarian explants of adult rats exposed to 0.034, 0.34 and 3.4 μM of DON for 24 h, a significant reduction of BCL-2 expression was accompanied by an increase of BAX and Caspase-3 expressions (Kolesárová et al., 2012). In addition, *in vitro* studies using sow granulosa cells reported that DON (500 to 2000 μg/L) results in a dose-dependent increase in the rate of apoptosis compared to the control group (Yang et al., 2020). A similar result was found in an *in vitro* study, where bovine granulosa cells exposure to DON (100 ng/mL) for 4 days showed an augmented proportion of apoptotic cells by 15% (Guerrero-Netro et al., 2015). Likewise, an increase in the expression levels of apoptotic proteins, such as Caspase-3, Caspase-9, poly (ADP-ribose) polymerase

(PARP), and the ratio of BAX / BCL-2 has also been observed in endometrial stromal cells of mice exposed to this mycotoxin (Dai et al., 2017). Due to its ability to inhibit protein synthesis through binding to the ribosome, DON is known to induce apoptosis in different cell types (Lucioli et al., 2013; Pestka and Smolinski, 2005).

In addition, although a relationship between mycotoxin exposure and impaired fertility has been suggested in the last few years (Eze et al., 2018), *in vivo* data on the literature reporting the effects of DON on ovaries are scarce (Yu et al., 2017). To the best of our knowledge, the present study is the first to analyze the apoptotic potential of DON on the ovaries using the *in vivo* model. Therefore, the present study demonstrated that activation of apoptotic signaling pathways in juvenile and peripubertal ovaries may be an important mechanism in reproductive toxicity of DON in *in vivo* model. Considering that estimation of dietary consumption of DON by children and adolescents may exceed the maximum temporary tolerable daily intake established by governmental agencies (Papageorgiou et al., 2018; Sundheim et al., 2017), future studies concerning the effects of DON in patients with puberty disorders are strongly encouraged.

5. Conclusions

This is the first study to evaluate the peripubertal exposure to DON on ovarian parameters. Our results indicate that activation of the apoptotic pathway in murine pubertal ovaries represent an important mechanism in the reproductive toxicity of DON. Available data in adult animals reveal a similar pattern suggesting that independently of the age, DON represents a reproductive disruptor. In this context, further studies including functional analysis and the identification of substances that can protect the animals of reproductive toxicity of DON are strongly recommended.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

Alm, H., Greising, T., Brüssow, K.P., Torner, H., Tiemann, U., 2002. The influence of the mycotoxins deoxynivalenol and zearalenol on in vitro maturation of pig oocytes and in vitro culture of pig zygotes. Toxicology in vitro: an international journal published in association with BIBRA 16, 643-648. DOI: 10.1016/s0887-2333(02)00059-0.

Andretta, I., Kipper, M., Lehnen, C.R., Hauschild, L., Vale, M.M., Lovatto, P.A., 2012. Meta-analytical study of productive and nutritional interactions of mycotoxins in growing pigs. Animal: an international journal of animal bioscience 6, 1476-1482. DOI: 10.1017/s1751731111002278.

Bracarense, A.P.F., Basso, K.M., Da Silva, E.O., Payros, D., Oswald, I.P., 2017. Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. World Mycotoxin Journal 10, 89-96. DOI: 10.3920/WMJ2016.2094.

Chuffa, L.G., Alves, M.S., Martinez, M., Camargo, I.C., Pinheiro, P.F., Domeniconi, R.F., Júnior, L.A., Martinez, F.E., 2016. Apoptosis is triggered by melatonin in an in vivo model of ovarian carcinoma. Endocrine-related cancer 23, 65-76. DOI: 10.1530/erc-15-0463.

Clark, E.S., Flannery, B.M., Pestka, J.J., 2015. Murine anorectic response to deoxynivalenol (vomitoxin) is sex-dependent. Toxins 7, 2845-2859. DOI: 10.3390/toxins7082845.

Dai, Y., Xie, H., Xu, Y., 2017. Evaluation of deoxynivalenol-induced toxic effects on mouse endometrial stromal cells: Cell apoptosis and cell cycle. Biochemical and biophysical research communications 483, 572-577. DOI: 10.1016/j.bbrc.2016.12.103.

De Santis, B., Debegnach, F., Miano, B., Moretti, G., Sonego, E., Chiaretti, A., Buonsenso, D., Brera, C., 2019. Determination of Deoxynivalenol Biomarkers in Italian Urine Samples. Toxins 11, 441. DOI: 10.3390/toxins11080441.

Deng, C., Li, C., Zhou, S., Wang, X., Xu, H., Wang, D., Gong, Y.Y., Routledge, M.N., Zhao, Y., Wu, Y., 2018. Risk assessment of deoxynivalenol in high-risk area of China by human biomonitoring using an improved high throughput UPLC-MS/MS method. Scientific Reports 8, 3901. DOI: 10.1038/s41598-018-22206-y.

Eze, U., Routledge, M., Okonofua, F., Huntriss, J., Gong, Y., 2018. Mycotoxin exposure and adverse reproductive health outcomes in Africa: a review. World Mycotoxin Journal 11, 321-339. DOI: 10.3920/WMJ2017.2261.

Friend, D.W., Trenholm, H.L., Fiser, P.S., Hartin, K.E., Thompson, B.K., 1986. EFFECT OF FEEDING DIETS CONTAINING DEOXYNIVALENOL (VOMITOXIN)-CONTAMINATED WHEAT OR CORN ON THE FEED CONSUMPTION, WEIGHT GAIN, ORGAN WEIGHT AND SEXUAL DEVELOPMENT OF MALE AND FEMALE PIGS. Canadian Journal of Animal Science 66, 765-775. DOI: 10.4141/cjas86-083.

Gerez, J.R., Camacho, T., Brunaldi Marutani, V.H., Nascimento de Matos, R.L., Hohmann, M.S., Verri Júnior, W.A., Bracarense, A., 2021. Ovarian toxicity by fusariotoxins in pigs: Does it imply in oxidative stress? Theriogenology 165, 84-91. DOI: 10.1016/j.theriogenology.2021.02.003.

Gerez, J.R., Desto, S.S., Bracarense, A.P.F.R.L., 2017. Deoxynivalenol induces toxic effects in the ovaries of pigs: An ex vivo approach. Theriogenology 90, 94-100. DOI: 10.1016/j.theriogenology.2016.10.023.

Grenier, B., Applegate, T.J., 2013. Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals. Toxins (Basel) 5, 396-430. DOI: 10.3390/toxins5020396.

Guerrero-Netro, H.M., Chorfi, Y., Price, C.A., 2015. Effects of the mycotoxin deoxynivalenol on steroidogenesis and apoptosis in granulosa cells. Reproduction (Cambridge, England) 149, 555-561. DOI: 10.1530/rep-15-0018.

Han, J., Wang, Q.C., Zhu, C.C., Liu, J., Zhang, Y., Cui, X.S., Kim, N.H., Sun, S.C., 2016. Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during porcine oocyte maturation. Toxicology and applied pharmacology 300, 70-76. DOI: 10.1016/j.taap.2016.03.006.

Iverson, F., Armstrong, C., Nera, E., Truelove, J., Fernie, S., Scott, P., Stapley, R., Hayward, S., Gunner, S., 1995. Chronic feeding study of deoxynivalenol in B6C3F1 male and female mice. Teratogenesis, carcinogenesis, and mutagenesis 15, 283-306. DOI: 10.1002/(sici)1520-6866(1996)15:6<283::aid-tcm5>3.0.co;2-e.

Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Grasl-Kraupp, B., 2017. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA Journal 15, e04718. DOI: 10.2903/j.efsa.2017.4718.

Kolesárová, A., Capcarová, M., Maruniaková, N., Baková, Z., Toman, R., Nath, S., Sirotkin, A.V., 2012. Deoxynivalenol-induced animal ovarian signaling: proliferation and apoptosis. Journal of Microbiology, Biotechnology and Food Sciences 9, 323-332.

Laffan, S.B., Posobiec, L.M., Uhl, J.E., Vidal, J.D., 2018. Species Comparison of Postnatal Development of the Female Reproductive System. Birth defects research 110, 163-189. DOI: 10.1002/bdr2.1132.

Lan, M., Han, J., Pan, M.H., Wan, X., Pan, Z.N., Sun, S.C., 2018. Melatonin protects against defects induced by deoxynivalenol during mouse oocyte maturation. Journal of pineal research 65, e12477. DOI: 10.1111/jpi.12477.

Lattanzio, V.M.T., Solfrizzo, M., De Girolamo, A., Chulze, S.N., Torres, A.M., Visconti, A., 2011. LC–MS/MS characterization of the urinary excretion profile of the mycotoxin deoxynivalenol in human and rat. Journal of Chromatography B 879, 707-715. DOI: 10.1016/j.jchromb.2011.01.029.

Liew, S.H., Nguyen, Q.-N., Strasser, A., Findlay, J.K., Hutt, K.J., 2017. The ovarian reserve is depleted during puberty in a hormonally driven process dependent on the pro-apoptotic protein BMF. Cell Death & Disease 8, e2971-e2971. DOI: 10.1038/cddis.2017.361.

Lucioli, J., Pinton, P., Callu, P., Laffitte, J., Grosjean, F., Kolf-Clauw, M., Oswald, I.P., Bracarense, A.P., 2013. The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: interest of ex vivo models as an alternative to in vivo experiments. Toxicon : official journal of the International Society on Toxinology 66, 31-36. DOI: 10.1016/j.toxicon.2013.01.024.

Malekinejad, H., Schoevers, E.J., Daemen, I.J., Zijlstra, C., Colenbrander, B., Fink-Gremmels, J., Roelen, B.A., 2007. Exposure of oocytes to the Fusarium toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. Biology of reproduction 77, 840-847. DOI: 10.1095/biolreprod.107.062711.

Maranghi, F., Mantovani, A., 2012. Targeted toxicological testing to investigate the role of endocrine disrupters in puberty disorders. Reproductive toxicology (Elmsford, N.Y.) 33, 290-296. DOI: 10.1016/j.reprotox.2012.01.009.

Massart, F., Meucci, V., Saggese, G., Soldani, G., 2008. High growth rate of girls with precocious puberty exposed to estrogenic mycotoxins. The Journal of pediatrics 152, 690-695, 695.e691. DOI: 10.1016/j.jpeds.2007.10.020.

Massart, F., Saggese, G., 2010. Oestrogenic mycotoxin exposures and precocious pubertal development. International journal of andrology 33, 369-376. DOI: 10.1111/j.1365-2605.2009.01009.x.

Medvedova, M., Kolesarova, A., Capcarova, M., Labuda, R., Sirotkin, A.V., Kovacik, J., Bulla, J., 2011. The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells in vitro. Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes 46, 213-219. DOI: 10.1080/03601234.2011.540205.

Mishra, S., Srivastava, S., Dewangan, J., Divakar, A., Kumar Rath, S., 2020. Global occurrence of deoxynivalenol in food commodities and exposure risk assessment in humans in the last decade: a survey. Critical Reviews in Food Science and Nutrition 60, 1346-1374. DOI: 10.1080/10408398.2019.1571479.

Morrissey, R.E., 1984. Teratological study of Fischer rats fed diet containing added vomitoxin. Food and Chemical Toxicology 22, 453-457. DOI: https://doi.org/10.1016/0278-6915(84)90328-4.

Morrissey, R.E., Vesonder, R.F., 1985. Effect of deoxynivalenol (vomitoxin) on fertility, pregnancy, and postnatal development of Sprague-Dawley rats. Applied and environmental microbiology 49, 1062-1066. DOI: 10.1128/aem.49.5.1062-1066.1985.

Papageorgiou, M., Wells, L., Williams, C., White, K., De Santis, B., Liu, Y., Debegnach, F., Miano, B., Moretti, G., Greetham, S., Brera, C., Atkin, S.L., Hardie, L.J., Sathyapalan, T., 2018. Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents. Toxins (Basel) 10. DOI: 10.3390/toxins10020050.

Pedersen, T., Peters, H., 1968. Proposal for a classification of oocytes and follicles in the mouse ovary. Reproduction (Cambridge, England) 17, 555-557. DOI: 10.1530/jrf.0.0170555.

Pestka, J.J., Smolinski, A.T., 2005. Deoxynivalenol: toxicology and potential effects on humans. Journal of toxicology and environmental health. Part B, Critical reviews 8, 39-69. DOI: 10.1080/10937400590889458.

Ranzenigo, G., Caloni, F., Cremonesi, F., Aad, P.Y., Spicer, L.J., 2008. Effects of Fusarium mycotoxins on steroid production by porcine granulosa cells. Animal reproduction science 107, 115-130. DOI: 10.1016/j.anireprosci.2007.06.023.

Rotter, B.A., Thompson, B.K., Rotter, R.G., 1994. Optimization of the mouse bioassay for deoxynivalenol as an alternative to large animal studies. Bulletin of environmental contamination and toxicology 53, 642-647. DOI: 10.1007/bf00196934.

Schoevers, E.J., Fink-Gremmels, J., Colenbrander, B., Roelen, B.A.J., 2010. Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. Theriogenology 74, 968-978. DOI: 10.1016/j.theriogenology.2010.04.026.

Silva, M.V., Pante, G.C., Romoli, J.C.Z., de Souza, A.P.M., Rocha, G., Ferreira, F.D., Feijó, A.L.R., Moscardi, S.M.P., de Paula, K.R., Bando, E., Nerilo, S.B., Machinski, M., Jr., 2018. Occurrence and risk assessment of population exposed to deoxynivalenol in foods derived from wheat flour in Brazil. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 35, 546-554. DOI: 10.1080/19440049.2017.1411613.

Sundheim, L., Lillegaard, I.T., Fæste, C.K., Brantsæter, A.-L., Brodal, G., Eriksen, G.S., 2017. Deoxynivalenol Exposure in Norway, Risk Assessments for Different Human Age Groups. Toxins 9, 46. DOI: 10.3390/toxins9020046.

Trenholm, H.L., Thompson, B.K., Foster, B.C., Charmley, L.L., Hartin, K.E., Coppock, R.W., Albassam, M.A., 1994. Effects of feeding diets containing Fusarium (naturally) contaminated wheat or pure deoxynivalenol (DON) in growing pigs. Canadian Journal of Animal Science 74, 361-369. DOI: 10.4141/cjas94-049.

Yang, M., Wu, X., Zhang, W., Ye, P., Wang, Y., Zhu, W., Tao, Q., Xu, Y., Shang, J., Zhao, D., 2020. Transcriptional analysis of deoxynivalenol-induced apoptosis of sow ovarian granulosa cell. Reproduction in Domestic Animals 55, 217-228. DOI: 10.1111/rda.13610.

Yang, R., Wang, Y.-M., Zhang, L.-S., Zhang, L., Zhao, Z.-M., Zhao, J., Peng, S.-Q., 2015. Delay of the onset of puberty in female rats by prepubertal exposure to T-2 toxin. Toxins 7, 4668-4683. DOI: 10.3390/toxins7114668.

Yu, M., Chen, L., Peng, Z., Nüssler, A.K., Wu, Q., Liu, L., Yang, W., 2017. Mechanism of deoxynivalenol effects on the reproductive system and fetus malformation: Current status and future challenges. Toxicology in Vitro 41, 150-158. DOI: 10.1016/j.tiv.2017.02.011.

4.4 Animal performance and clinical response to deoxynivalenol is sex-dependent in peripubertal rats

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Abstract

Deoxynivalenol (DON), a mycotoxin produced mainly by *Fusarium graminearum* and *F. culmorum* commonly contaminates food commodities across the globe. Due to this, exposure to DON might pose potential health hazards to humans and animals. Biological factors like sex and age can influence the toxicity of DON. However, in toxicological studies involving DON, the sex and age-dependent response has been often overlooked. Thereby the objective of this study was to evaluate if sex differences are similarly evident in DON's systemic effects in pubertal rats. Juvenile animals (n = 24) with 28 days postnatal day were randomly assigned to two experimental groups: Control group (n = 12, 6 females and 6 males, mycotoxin-free diet) and DON group (n = 12, 6 females and 6 males, diet containing 10 mg DON/kg of feed). During 28 days of treatment, the animals were weighed weekly and body weight gain and food intake were calculated for each week. After the experimental period, blood samples, intestine, liver, and kidney were collected and destined for biochemical, hematological, histopathological, and oxidative stress analyses. Greater anorectic responses were seen in male than female rats, while only females showed increased levels of creatinine and triglycerides. Independently of sex, DON induces an increased number of lymphocytes and leucocytes with a significant reduction in the levels of hemoglobin, hematocrit, mean corpuscular hemoglobin, and neutrophils. In males and females fed a DON-contaminated diet, histological lesions were observed in the intestine, liver, and kidney. Ingestion of DON induced a significant increase in the antioxidant potential in the intestine, liver, and kidney. However, this effect was not able to prevent oxidative stress in the renal tissue. Taken together, our results showed a sex-related response in food intake, weight gain, and biochemical parameters in rats exposed to DON during the juvenile and peripubertal periods. In addition, we have verified that oxidative stress is an important mechanism in the nephrotoxicity of DON.

Keywords trichothecenes, sex dependence, histology, oxidative stress

Introduction

Deoxynivalenol (DON) is a mycotoxin produced mainly by *Fusarium graminearum* and *F. culmorum* that commonly contaminate food commodities across the globe. Due to this, exposure to DON might pose potential health hazards to humans and animals (Mishra et al. 2019; Pestka 2010b). Upon consumption of contaminated feed or food, acute effects including vomiting, abdominal pain, diarrhea, and fever were reported, whereas chronic intoxication with low doses has been characterized by anorexia, growth retardation, immunotoxicity as well as reproductive, developmental, and neuronal abnormalities (Pestka 2010b). The toxic effects of DON have been observed in different animal species in the following order of susceptibility swine > mice > rats > poultry ≈ ruminants (Eriksen and Pettersson 2004).

At the cellular level, DON binding to ribosomes can rapidly activate mitogen-activated protein kinases (MAPKs) and induce the inflammatory cytokine and chemokine expression as well as apoptosis (Shifrin and Anderson 1999). In addition, in the last decade studies indicate the role of oxidative stress in DON mediated toxicity. In *in vitro* studies, DON generates reactive oxygen species (ROS), modulating the intracellular antioxidant systems, promoting lipid peroxidation, and ultimately apoptosis (Wu et al. 2014). However, *in vivo* studies that evaluate the role of oxidative stress in the toxicity of DON are scarce and extremely important since *in vitro* models do not mimic the complex pattern of cell differentiation present in tissues *in vivo* (Hou et al. 2013; Mishra et al. 2014; Szabó et al. 2018).

Additionally, biological factors like sex and age can influence the toxicity of DON. Male mice compared to females upon acute DON exposure eliminate DON more slowly, exhibiting higher concentrations of IL-6 and DON in organs and plasma (Clark et al. 2015; Pestka et al. 2017). A similar result was observed previously, where the urinary biomarker analysis of human volunteers exposed to a single oral dose of DON after 24 hours (1 µg/kg body weight) revealed that women excrete more quickly DON than men (Vidal et al. 2018). In this way, a higher susceptibility of males versus females to DON-induced anorectic effects has been observed in mice and pigs (Andretta et al. 2012; Clark et al. 2015; Cote et al. 1985; Iverson et al. 1995; Pestka et al. 2017). In addition, sex-dependent effects of DON have been described in the immune response of mice exposed to dietary DON (Wu et al. 2009). According to previous studies, males are more susceptible to DON-induced IgA nephropathy than female mice (Greene et al. 1995; Greene et al. 1994a; Greene et al. 1994b). In contrast, a reduced number of peripheral blood monocytes and splenic macrophages were observed in female mice but not in male mice (Iverson et al. 1995; Wu et al. 2009). Nevertheless, toxicological studies about the sex-specific effects of DON in animals are scarce (Soler and Oswald 2018).

Besides these aspects, in some countries, higher levels of DON have been observed in the urine samples of children and adolescents in comparison to adults (Brera et al. 2015; Papageorgiou et al. 2018b). Also, when the concentration of DON in urine and food was used to estimate the mean intake of DON by children and adolescents, the values obtained exceeded the provisional maximum tolerable daily intake (PMTDI) established by Joint Food and Agriculture Organization (FAO) / WHO Expert Committee on Food Additives (JECFA) (1 µg / kg b.w./day) (Brera et al. 2015; JECFA 2001; JECFA 2011; Papageorgiou et al. 2018b; Silva et al. 2018b). In mice, age-related differences were also reported. Weaned mice exposed to a single oral dose of DON (5mg/kg) presented higher concentrations of DON in plasma, spleen, and kidneys and increased expression of cytokines when compared to adult mice (Pestka and Amuzie 2008). However, to the best of our knowledge, *in vivo* studies on the systemic toxicity of DON on juvenile-peripubertal transition period have been overlooked in toxicological research. Therefore, the purpose of this study was to evaluate sex differences in animal performance, biochemical, hematological, and histological parameters using rats submitted to chronic ingestion of DON-contaminated feed during the juvenile and peripubertal periods. The role of oxidative stress in DON intestinal, hepatic, and renal toxicity was also evaluated.

Material and methods

Animals and diet

Twelve juvenile female and male Wistar rats, 21-days-old, were carefully kept under pathogen-free conditions and housed in adequate conditions for seven days before the beginning of the experiment. The animals were fed a standard diet (Quimtia®) and water *ad libitum*. During the experiment, the animals were kept in a room with adequate temperature for thermal comfort (± 21°C), adequate ventilation (exhaustion of air), humidity (40-70%), and photoperiod (12h of light and 12h of dark). The project was previously presented and approved by the Animal Use Ethics Committee of our institution (CEUA protocol nº 6986.2017.27).

Experimental design

Juvenile animals (n = 24) with 28 days postnatal day (Ojeda et al. 1980) were randomly assigned to two experimental groups: Control group (n = 12 – 6 females and 6 males, mycotoxin-free diet) and DON group (n = 12 - 6 females and 6 males, diet containing 10 mg DON/kg of feed). A crude extract of DON (40 mg/kg) produced at the Laboratory of Mycology, Luiz de Queiroz College of Agriculture, Universidade de São Paulo, was used in the preparation of the contaminated diet. A blend (standard diet+DON) was prepared at Universidade Estadual de Londrina facilities using a commercial feed mixer. The diets were sent to the Samitec laboratory (Santa Maria – RS/Brazil), where the mycotoxins levels were assessed using the HPLC/MS-MS method. The control diet showed lower levels of DON than the limit of quantification established (200 µg/kg with a stated accuracy of 101%). According to Grenier and Applegate (2013), the dose of DON used in the treatment (10 mg/kg of feed) corresponds to a dose occasionally found in commodities, feeds, and feed ingredients However, a realistic dose was not chosen since a higher metabolism of DON to DOM-1 is found in rats when compared to humans (Lattanzio et al. 2011). In addition, the chosen dose is in agreement with other relevant studies that have evaluated the toxicity of DON in the murine species (Bracarense et al. 2017; Clark et al. 2015). The animals were fed with a control or a contaminated diet for 28 days. Thus, after the experimental period, euthanasia was performed using an intraperitoneal overdose of sodium pentobarbital, which was confirmed by dislocation of the cervical segment of the spinal cord and decapitation. Then, the blood samples were collected by cardiac puncture and destined for biochemical and hematological analyses. In addition, histopathological and oxidative stress parameters were evaluated in samples of jejunum, liver, and kidney.

Zootechnical parameters

During the experiment, two animals of the same sex were kept per cage, and for the zootechnical analysis, the cage was considered the experimental unit. The animals were weighed weekly and body weight gain and food intake were calculated for each week.

Hematology and biochemistry

A complete blood count (CBC) was performed immediately after blood collection in an automated system, poCH-100*iV Diff*™ (Sysmex, Kobe, Japan) for red blood cells (RBC) and white blood cells (WBC) counts, hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). WBC differential counts and manual platelet (PLT) estimation to confirm electronic PLT counts were performed on blood smears stained with Diff-Quick (NEWPROV, Paraná, Brazil).

The biochemical tests were performed on Dimension® Xpand Plus clinical chemistry system (SIEMENS, Siemens Healthcare Diagnostics Inc., PA, USA) to determine the following plasma analyses: creatinine, urea, uric acid, glucose, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, high-density lipoprotein (HDL), and triglycerides.

Histology

Tissue fragments of the jejunum, liver (medial part of the left lobe), and left kidney were collected from 8 animals (4 males and 4 females). Following these fragments were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3 μm were stained with hematoxylin–eosin (HE) for histopathological evaluation. For each organ, three slides per animal were prepared for analysis, and an area of 2000–2500 mm^2 per slide was observed.

For analysis of each organ, a morphological score was used according to Bracarense et al. (2017) and Luo et al. (2019), where the frequency and severity of each criterion were considered by using the predetermined score: 0 (absent), 1 (discrete), 2 (moderate), and 3 (severe).

Oxidative stress response analysis

Samples (n=4/group) of jejunum, liver, and kidney were collected in sterile microtubes, maintained at −80 °C. For measuring the antioxidant capacity, the tests of reduced glutathione (GSH) according to Sedlak and Lindsay (1968), ferric reducing ability (FRAP) and reducing of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) following the methodologies described by Katalinic et al. (2005) were performed. The superoxide anion production was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) (Fattori et al. 2015) and lipid peroxidation was assessed by the levels of thiobarbituric acid reactive substances (TBARS) (Manchope et al. 2018). Briefly, using a tissue tearor (Bjospec, São Paulo, SP, Brazil) the samples were homogenized in KCL (1.15%) buffer for FRAP, ABTS, NBT, and TBARS, and EDTA buffer (0.02 M) for GSH test, followed by the protocol described by the authors cited above.

Statistical analysis

The data were represented as means±SD (standard deviation) and analyzed using the GraphPad Prism Software. First, the assumptions of residual normality (Shapiro's test) and homoscedasticity (Bartlett's test) were confirmed. Following, the factorial ANOVA was used when the research question asks for the influence of two independent variables (treatment and sex) on one dependent variable (zootechnical, biochemical, hematological, and histological parameters) ($p \leq 0.05$). For evaluation of treatment on oxidative stress response the Student's t-test was used ($p \leq 0.05$).

Results

Sex-related effect of DON on animal performance

We first investigated the sex-dependent effect of DON on the growth performance of peripubertal rats (Table 1). Animals fed the DON-diet showed an interaction among treatment x sex in the final body weight (p=0.002), weight gain (0 to 14 days / p=0.03; 15 to 28 days / p=0.006) and food intake (15 to 28 days / p=0.007). In males, DON induced a reduction (12%) in the final body weight when compared with control males (p=0.003). After 14 days, male rats fed the DON-contaminated diet exhibited a decrease (29.5%) in the body weight gain compared to control males (p=0.006), while no change was observed in the food intake during the same period (p=0.40). In addition, during the last 14 days, male rats exposed to DON showed a significant reduction in weight gain (17.4%, p=0.03) and suppression in food intake (22.1%, p=0.01) in relation to the control males. By contrast, in female rats, DON treatment did not induce any changes in the final body weight, weight gain, and food intake.

Table 1 Sex-related effect of deoxynivalenol (DON) on animal performance in rats[a)]

Item	Control ♂	DON ♂	Control ♀	DON ♀	Control ♀♂	DON ♀♂	P-value		
							Treat[1]	Sex	Treat*Sex
Initial BW[2] (g)	185.00±5.00a	191.67±7.64a	168.33±7.64a	176.67±2.89a	176.70±10.80A	184.20±9.70A	0.10	0.0021	0.82
Final BW	481.67±22.55a	423.33±7.64b	325.00±5.00a	338.33±10.41a	403.30±78.33A	380.80±42.50B	0.02	<0.0001	0.002
Days 0-14									
WG[3] (g)	146.67±18.93a	103.33±11.54b	88.33±2.88a	81.67±7.63a	117.50±34.17A	92.50±27.64B	0.006	0.0004	0.03
FI[5] (g)	531.23±26.04a	545.92±13.54a	429.97±19.80a	449.65±42.03a	480.60±54.04A	497.55±54.17A	0.32	0.0003	0.86
Days 15-28									
WG (g)	153.33±5.77a	126.67±15.27b	68.33±7.63a	80.00±0.00a	109.17±45.43A	104.20±27.64A	0.18	<0.0001	0.006
FI[4] (g)	652.38±41.07a	508.13±63.30b	454.99±7.60a	485.24±33.96a	553.68±101.59A	496.68±43.02B	0.04	0.002	0.007

[a)] Results are expressed as mean±SD. [1]Treat: treatment; [2]BW: body weight; [3]WG: weight gain; [4]FI: feed intake; [a, b] different lower-case letters indicate statistical significance between the treatments within each sex by factorial ANOVA (2x2) p<0.05 followed by Student's t-test p≤0.05; [A, B] different capital letters indicate statistical significance between the diets independently of the sex by factorial ANOVA (2x2) p<0.05.

Biochemical and hematological responses to DON is sex-dependent

At the end of the experiment, blood samples were collected to investigate the sex-related effects of DON on biochemical and hematological parameters (Table 2). A significant interaction between treatment and sex on the creatinine and triglycerides levels (p=0.04; p=0.01) was detected. In females, DON induced a significant increase in the creatinine (3.2 fold increase, p=0.03) and triglycerides (1.6 fold increase, p=0.03) concentrations compared to control females. On the other hand, males did not display significant changes in these parameters. In addition, ingestion of a contaminated diet, independently of the sex, induced a significant decrease in ALP (1.3 fold decrease, p=0.02), an increase in the cholesterol (1.2 fold increase, p=0.05), and high-density lipoprotein levels (1.16 fold increase, p=0.009) compared to control animals. No other significant effect of DON on biochemical parameters was observed.

Table 2 Effects of deoxynivalenol (DON) on biochemical parameters of pubertal male and female rats a)

Item	Control ♂	DON ♂	Control ♀	DON ♀	Control ♀♂	DON ♀♂	P-value		
							Treat[1]	Sex	Treat*Sex
ALT[2] (U/L)	43.20± 4.76a	54.25± 7.63a	46.00± 4.18a	44.80± 13.74a	44.60± 1.40A	49.52± 4.72A	0.22	0.41	0.14
AST[3] (U/L)	120.50± 44.33a	112.50± 23.47a	154.00± 50.14a	110.80± 21.84a	137.25± 16.75A	111.65± 0.85A	0.16	0.37	0.32
ALP[4] (U/L)	219.00± 31.04a	175.00± 51.54a	161.25± 22.23a	127.00± 18.07a	190.12± 28.87A	151.00± 24.00B	0.02	0.004	0.75
Glucose (mg/dL)	192.50± 41.19a	191.50± 9.04a	160.40± 21.50a	188.20± 35.63a	176.45± 16.05A	186.85± 1.65A	0.36	0.23	0.32
Creatinine (mg/dL)	0.27± 0.06a	0.27± 0.02a	0.10± 0.05a	0.32± 0.04b	0.18± 0.08A	0.29± 0.02B	0.03	0.23	0.04
Urea (mg/dL)	48.33± 6.86a	46.00± 4.97a	44.60± 5.50a	49.60± 4.10a	46.47± 1.87A	47.80± 1.80A	0.60	0.97	0.16
Uric acid (mg/dL)	2.07± 1.81a	2.23± 0.68a	2.02± 0.50a	2.40± 1.60a	2.04± 0.02A	2.31± 0.09A	0.66	0.92	0.86
Cholesterol (mg/dL)	78.33± 11.72a	81.50± 6.75a	91.80± 12.85a	108.80± 9.09a	85.07± 6.73A	96.87± 15.37B	0.05	0.0006	0.17
HDL[5] (mg/dL)	59.17± 9.15a	65.25± 4.27a	72.25± 6.85a	87.20± 7.92a	65.71± 6.54A	76.22± 10.97B	0.009	0.0002	0.22
Triglycerides (mg/dL)	48.60± 13.13a	41.75± 12.66a	41.60± 12.70a	64.60± 8.56b	45.10± 3.50A	53.17± 11.42A	0.16	0.17	0.01

a) Results are expressed as mean±SD. [1]Treat: treatment; [2]ALT: alanine aminotransferase; [3]AST: aspartate aminotransferase; [4]ALP: alkaline phosphatase; [5]HDL: high density lipoprotein. [a, b] different lower-case letters indicate statistical significance between the treatments within each sex by factorial ANOVA (2x2) $p<0.01$ followed by Student's t-test $p \le 0.05$; [A, B] different capital letters indicate statistical significance between the diets independently of the sex by factorial ANOVA (2x2) $p<0.05$.

The results of hematological parameters are disposed in Table 3. Ingestion of DON contaminated feed resulted in a significant decrease in the hemoglobin (1.96 fold decrease, p<0.0001), hematocrit (1.07 fold decrease, p=0.03), mean corpuscular volume (1.02, p= 0.003), mean corpuscular hemoglobin (1.02 fold decrease, p=0.05), and number of neutrophils (1.78 fold decrease, p=0.0006), independently of the sex. In addition, the animals exposed to DON showed a significant increase in the white blood cells (1.28 fold increase, p=0.03), and lymphocytes number (1.46 fold increase, p=0.002) compared to control animals.

Table 3 Effects of deoxynivalenol (DON) on hematological parameters of male and female rats[a)]

Item	Control ♂	DON ♂	Control ♀	DON ♀	Control ♀♂	DON ♀♂	P-value		
							Treat[1]	Sex	Treat*Sex
RBC[2] (10^6/mm^3)	8.21± 0.62	8.11± 0.36	8.74± 0.74	8.21± 0.62	8.47± 0.27	8.16± 0.05	0.23	0.23	0.40
HGB[3] (g/dL)	15.43± 8.23	8.23± 0.33	16.35± 1.51	8.21± 0.42	15.89± 0.46	8.22± 0.01*	<0.0001	0.30	0.28
HCT[4] (%)	47.20± 1.11	46.20± 1.37	49.95± 2.17	44.77± 0.87	48.57± 1.37	45.48 0.71*	0.03	0.63	0.14
MCV[5] (fL)	57.53± 1.21	56.95± 1.07	57.12± 0.78	54.53± 0.79	57.33± 0.20	55.74± 1.21*	0.003	0.007	0.06
MCH[6] (pg)	18.82± 0.60	18.57± 0.29	18.70± 0.32	18.12± 0.35	18.76± 0.06	18.35± 0.23*	0.05	10.17	0.40
MCHC[7] (g/dL)	32.70± 0.37	32.62± 0.29	32.72± 0.21	33.20± 0.37	32.72± 0.01	32.91± 0.28	0.20	0.06	0.09
RDW[8] (%)	10.12± 0.99	9.75± 0.63	9.17± 0.17	9.73± 0.95	9.65± 0.47	9.74± 0.008	0.80	0.22	0.23
WBC[9] (10^3/mm^3)	4.76± 1.42	7.17± 8.69	6.00± 1.93	6.63± 1.22	5.38± 6.20	6.90± 2.71*	0.03	0.59	0.18
Band neutrophils (mm^3)	0.00 0.00	0.00 0.00	19.75± 19.75	0.00 0.00	7.90± 7.90	0.00 0.00	0.24	0.24	0.24
Segmented neutrophils (mm^3)	968.83± 497.28	705.00 188.09	1480.25± 746.55	667.40± 212.97	1224.54± 255.71	686.20± 18.80*	0.03	0.32	0.25
Lymphocytes (mm^3)	4179.17± 1254.53	6161.50± 996.94	4411.25± 1436.03	6333.80± 751.18	4272.00± 116.04	6257.22± 86.15*	0.002	0.70	0.95
Eosinophils (mm^3)	102.00± 60.55	66.75± 25.16	88.75± 35.84	43.40± 30.22	96.70± 6.62	53.78± 11.67	0.40	0.70	0.91
Monocytes (mm^3)	0.00 0.00	19.00± 19.00	0.00 0.00	15.40± 15.40	0.00 0.00	17.00± 17.00	0.15	0.87	0.87
Platelets (mm^3)	1062500± 72104	1056500± 98072	1123250± 98973	1173833± 67483	1092875± 30375	1115167± 58666	0.79	0.30	0.74

a) Results are expressed as mean±SD. [1]Treat: treatment; [2]RBC: red blood cells; [3]HGB: hemoglobin; [4]HCT: hematocrit; [5]MCV: mean corpuscular volume; [6]MCH: mean corpuscular hemoglobin; [7]MCHC: mean corpuscular hemoglobin concentration; [8]RDW: red cells distribution width; [9]WBC: white blood cells; *indicate statistical significance between the diets independently of the sex by factorial ANOVA (2x2) p<0.05.

Sex-related effect of DON on the histology of intestine, liver, and kidney

We were interested in investigating if DON shows a sex-dependent response on the histological aspects of the intestine, liver, and kidney. No histological changes were observed in the organs of rats fed the control diet (Figure 1 A, C, and E). DON exposure induced a significant increase in the morphological score (Table 4); however, no sex interaction was observed.

In the intestine, DON induced moderate multifocal atrophy and fusion of villi, lymphatic vessel dilation, and flattening of epithelial cells (Figure 1B). As indicated by the morphological score, rats fed the DON-contaminated diet displayed significant jejunal lesions (a 1.63-fold increase) when compared with animals fed the control diet (p=0.0004) (Table 4). The hepatic lesion in animals exposed to DON demonstrated discrete trabecular disorganization, moderate cytoplasmic vacuolization of hepatocytes, and necrosis of hepatocytes (Figure 1D). The morphological score was elevated 1.14-fold higher after DON treatment when compared to control animals (p=0.0009).

Additionally, in the kidneys, the morphological score significantly increases (3.09-fold) with a diet containing DON (Table 4). The main histological changes in the kidneys were cytoplasmic vacuolization of tubular epithelial cells, interstitial infiltrate of lymphocytes (focal pattern), and apoptosis of renal tubular epithelial cells (Figure 1F).

Table 4 Effects of deoxynivalenol (DON) on the morphological score of intestine, liver, and kidneys of pubertal male and female rats$^{a)}$

Morphological score (A.U.)	**Control**	**DON**	**Control**	**DON**	**Control**	**DON**	**P-value**		
	♂	♂	♀	♀	♀♂	♀♂	Treat^1	Sex	Treat*Sex
Intestine	4.20± 0.20	6.75± 0.75	3.00± 0.58	5.25± 0.48	3.67± 0.33A	6.00± 0.50B	0.0004	0.02	0.77
Liver	1.50± 0.64	5.25± 0.75	1.25± 0.48	6.33± 2.03	1.38± 0.37A	5.71± 0.89B	0.0009	0.68	0.51
Kidney	2.25± 0.25	4.67± 0.33	1.00± 0.58	5.25± 1.03	1.62± 0.37A	5.00± 0.58B	0.0004	0.63	0.20

a) Results are expressed as mean±SEM. ^{1}Arbitray units. $^{A, B}$ Different capital letter indicates the statistical difference between the diets independently of the sex by factorial ANOVA (2x2) P<0.001

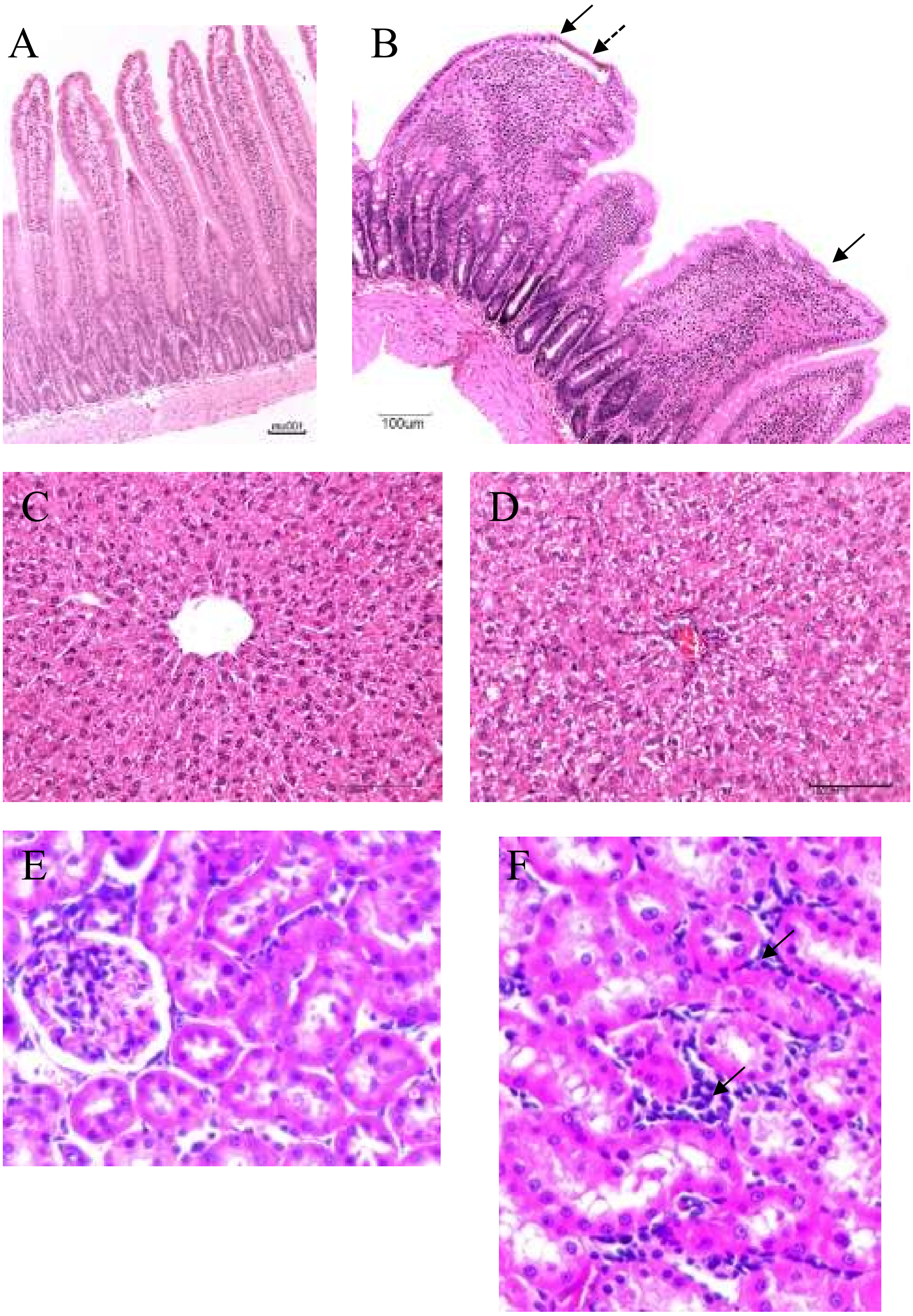

Figure 1. Effects of deoxynivalenol (DON) on jejunum, liver, and kidney. A) Jejunum of a control rat with normal villi (HE, 4×). B) Jejunum of a DON-treated rat with villi flattening and fusion (arrow) and villi showing flattened epithelial cells (dotted arrow) (HE, 4x,). C) N*ormal* liver tissue of control rat (HE, 10x). D) Liver of a DON-treated rat. Disorganization of hepatic cords, cytoplasmic vacuolization, and necrosis of hepatocytes (HE, 10x). E) Normal renal tissue of control rat (HE, 20x). F) Kidney of a DON-treated rat. Infiltrate inflammatory (arrow) (HE, 10x).

Effect of DON on the oxidative stress of the intestine, liver, and kidney

Considering the previous results, DON-induced oxidative stress was investigated in a sex-independent manner through the antioxidant defense status (FRAP, ABTS, and GSH assays), lipid peroxidation (TBARS), and superoxide anion production (NBT) (Table 6).

DON significantly decreased the antioxidant defenses in the intestine as observed by reductions in ABTS and FRAP assays (p=0.0009; p=0.03). By contrast, GSH levels in the liver were significantly increased in animals exposed to DON (p=0.05). Additionally, in renal tissue, DON significantly increased the ABTS scavenging ability, but this effect was not able to prevent a significant increase in NBT and TBARS levels (Table 6).

Table 6 Effects of deoxynivalenol (DON) on oxidative stress in the intestine, liver, and kidneys of pubertal male and female rats[a)]

	GSH[1] (nmol/mg of protein)	ABTS[2] (nmol/Trolox/ mg of protein)	FRAP[3] (nmol TroloxEq/mg of protein)	NBT[4] reduction (OD/mg of protein)	TBARS[5] (ΔOD A_{535}-A_{532}/mg protein)
Intestine					
Control	16.28±3.21	6.59±0.14	21.40±1.42	3.47±0.18	0.50±0.15
DON	10.54±1.38 P=0.18	5.20±0.12* P=0.0009	16.54±0.19* P=0.03	2.78±0.37 P=0.14	0.41±0.05 P=0.61
Liver					
Control	9.72±1.29	4.09±0.12	20.97±2.41	1.73±0.11	0.28±0.04
DON	15.96±2.11* P=0.05	4.15±0.46 P=0.91	18.72±3.83 P=0.64	1.62±0.19 P=0.65	0.38±0.08 P=0.33
Kidney					
Control	11.39±1.04	3.17±0.32	16.65±1.01	1.23±0.10	0.31±0.08
DON	16.14±4.39 P=0.33	5.22±0.83* P=0.05	19.30±1.45 P=0.18	1.87±0.18* P=0.04	0.59±0.05* P=0.04

a) Results are expressed as mean±SEM. [1]GSH-reduced glutathione, [2]ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, [3]FRAP-ferric-reducing antioxidant power, [4]NBT-nitroblue tetrazolium (NBT) reduction assay, [5]TBARS-thiobarbituric acid reactive substances. * indicates statistical significance within a column between the diets by Student's t-test (P≤0.05).

Discussion

Sex- and age-related susceptibility to adverse effects of some mycotoxins have been described (Soler and Oswald 2018). Recent studies indicated that the estimated mean intakes of DON by children and adolescents exceed the stipulated PMTDI (Papageorgiou et al. 2018a; Silva et al. 2018a), and remarkably the toxicity of this mycotoxin on childhood and puberty has been poorly assessed. In this study, we have demonstrated the influence of sex in peripubertal rats fed a DON diet on food intake, weight gain, and some biochemical parameters. In addition, we have determined the oxidative stress response as an important mechanism of DON toxicity in peripubertal rats.

Initially, the effect of DON was evaluated on zootechnical parameters. At 14 and 28 days of treatment, a significant reduction in the final body weight, weight gain, and feed intake were observed in males fed a DON-contaminated diet, while female mice showed no difference. Similar results were reported by Clark et al. (2015) and Iverson et al. (1995) where adults male mice exposed to a diet contaminated with 10 mg/kg DON feed for 17 days and 2 years, respectively, presented a significant decrease in body weight, while female mice did not. Analogous effects were observed in piglets fed different levels of DON (0.7, 3.1, and 5.8 mg/kg of feed) and zearalenone (0.05 and 0.1 mg/kg of feed) for 4 weeks; males showed overall lower weight gain compared with females receiving the same diet (Cote et al. 1985). Moreover, a meta-analysis study found a greater mycotoxin effect on the growth of growing male pigs compared to the effect on females (Andretta et al. 2012). Interestingly, when the sex effect was evaluated on the metabolism and clearance of this toxin in mice, the increased male´s sensitivity to DON’s anorectic effects compared to females is associated with a decreased ability to eliminate the toxin via urine (Pestka et al. 2017).

Considering the hepatotoxicity and nephrotoxicity of DON observed in *in vivo* and in *vitro* previous studies (Bracarense et al. 2017; Kouadio et al. 2013; Liang et al. 2015; Sahu et al. 2010), biochemical analyses were carried out to evaluate lipid metabolism and biomarkers of hepatic and renal injury.

Studies evaluating biochemical and hematological parameters after DON exposure in rats are scarce. The present study brings interesting results regarding some of them. We observed a decrease in ALP activity, and an increase in cholesterol, HDL levels in the DON group. In addition, an increase in plasma triglycerides and creatinine only in females treated with DON was observed. The decrease in ALP activity was of small magnitude (1.26-fold), and can be correlated with the hepatic necrosis observed in the DON-treated animals (Thrall et al., 2015). An increase in plasma creatinine can indicate kidney damage, and although urea did not increase, urea is a less sensitive marker for kidney damage than creatinine (Kaneko et al., 2008). Therefore, the increase in creatinine associated with oxidative damage in the kidneys indicates possible kidney damage in this study. Another explanation for creatinine increasing in females is because growing animals increase their muscle mass and creatinine is the final product of creatine metabolism in the muscle cells (Kaneko et al., 2008). In the present study, females consumed more feed and gained more weight than males, consequently, this was expected. Concerning the lipidic profile, it was demonstrated that female rats have higher contents of HDL resulting from the positive effects of estrogen that increase HDL (Ihedioha et al., 2013). The effects of DON on biochemical parameters present contradictory results in different species. In male rats exposed subcutaneously to DON (1mg/kg b.w. for three days) no changes in blood cholesterol and triglycerides levels were observed (Szkudelska et al. 2002). This lack of changes has also been described in piglets (1 and 0.5 nmol toxin/kg b.w./day DON for 2-3 weeks) and 7-week-old rabbits (10mg/kg DON for 3 weeks) (Kachlek et al. 2017; Pierron et al. 2018). On the other hand, in chickens, an increase in serum triglycerides and cholesterol was described and associated with altered lipid metabolism (Azizi et al. 2021; Ghareeb et al. 2016). Therefore, our results indicate that sex influences feed consumption and consequently related biochemical parameters, such as creatinine. Furthermore, DON possibly exerts an effect on lipid metabolism in female peripubertal rats, as DON ingestion increases cholesterol and triglycerides as well as in chicken (Azizi et al., 2021; Ghareeb et al., 2016). This hypothesis is reinforced as females did not decrease their feed consumption regardless of whether the feed is contaminated with DON or not.

Regarding the hematological results in the present study, the decrease in HGB, HCT, and MCV in animals fed with DON indicates the presence of microcytic anemia due to iron deficiency. In inflammation, the inflammatory mediators prevent the use of iron by the bone marrow (Feldman et al., 2000). It was also observed a decrease in WBC because there was a decreasing number of segmented neutrophils, which probably migrated to tissues injured by DON (Feldman et al., 2000). For the increase in lymphocytes, lymphocytosis in rats occurs as a response to inflammation (Bolliger et al. 2010). Contradictory results were found in rats, of unknown sex and age, which received 1 intraperitoneal injection of 1 mg/kg DON, in which DON showed minimal hematotoxicity in relation to the other mycotoxins studied. It was reported a decrease in WBC, and also a decrease in lymphocytes (Chattopadhyay et al. 2013). This contradiction in lymphocytes can occur because DON has immunomodulatory activity, depending on the dose used (Pestka 2010a). Therefore, our results showed that, regardless of sex, the treatment with DON caused only mild hematological changes probably related to the inflammatory state caused by DON. However, it should be noted that most of the biochemical and hematological parameters were slightly affected by DON ingestion, as these parameters were still within their physiological reference range (He et al. 2017; Ihedioha et al. 2013), and therefore, of limited physiological relevance.

The sexual dimorphism of the toxic effect of DON on histopathology was observed through a morphological score. In males and females, DON induced intestinal, hepatic, and renal histological changes. In accordance, similar results were described in the intestine, liver, and kidney of male adults rats chronically fed with DON-contaminated diets (8.2 and 11.4 mg/kg feed) (Bracarense et al. 2017; Luo et al. 2019), and female adults mice exposed to four intraperitoneal doses (1.5, and 2.5 mg of DON /kg bw) after 5, 8, and 12 hours after the last inoculation (Liang et al. 2015). Additionally, in a chronic feeding study in male and female mice exposed to 2 years to DON the histological results provided statistically significant dose-related evidence for a decrease in liver preneoplastic and neoplastic lesions as the dose level of DON increased (Iverson et al. 1995). It is interesting to note that data on the literature reporting histological aspects of sexual dimorphism in the toxicity of DON is scarce. To the best of our knowledge, the present study is the first to analyze the importance of accounting for sex in the toxicity of DON on histological parameters of pubertal animals.

It is well established that DON at the cellular level induces the inhibition of protein and nucleic acid synthesis (Shifrin and Anderson 1999; Ueno et al. 1973) via binding to the ribosome and by activating mitogen-activated protein kinase (MAPKs) (Shifrin and Anderson 1999). However, in the past several years, there has been considerable focus on the potential of DON to induce oxidative stress (Da Silva et al. 2018). In previous studies, DON induces intestinal oxidative stress in piglets and cells culture (Da Silva et al. 2019; Kouadio et al. 2007; Kouadio et al. 2005; Zha et al. 2020). In this study, a significant reduction of the antioxidant capacity was observed in the intestine of rats exposed to DON, while no effect of the contaminated diet was observed in the superoxide and malondialdehyde (MDA) levels (TBARS assay). Swine are the animals more susceptible and this can explain the differences observed between the animal models (Eriksen and Pettersson 2004).

In the liver of animals fed with a DON-contaminated diet, a significant increase in GSH levels was observed. In contrast, in adults rats treated orally with DON (5 mg/kg body weight) for 21 days, hepatotoxicity was associated with lipid peroxidation and decreased glutathione (Abdel-Wahhab et al. 2015). This difference can probably be related to an important role of age-related changes in the functioning of antioxidant systems of hepatic cells (Conforto and Waxman 2012). Interestingly, in the acute hepatotoxicity, increased hepatic GSH level after pretreatment with vitamin E, did not significantly modify the genotoxicity of DON, indicating that oxidative stress may not be the only mechanism involved in DON hepatotoxicity. In this study, in the kidney, DON induced oxidative stress characterized by an increased level of superoxide and MDA. In accordance, apoptosis, dysfunction, and oxidative stress in mouse kidney was observed in animals exposed to acute intoxication (Liang et al. 2015). In addition, oxidative stress has been associated with nephrotoxicity of DON in piglets fed a diet contaminated (4 mg/kg of feed) for 37 days (Wu et al. 2014).

Conclusion

In conclusion, in this study, we have confirmed male predilection to DON-induced changes in food intake and weight gain in pubertal animals. Moreover, reflexes of the anorectic response observed only in males may influence biochemical analysis, but not hematological and histopathological parameters. Sex is not a determinant for deoxynivalenol toxicity in the hematological and histological parameters of pubertal rats. In addition, in pubertal animals, oxidative stress is an important mechanism in the nephrotoxicity of DON.

Conflict of interest statement

The authors declare no conflicts of interest.

References

Abdel-Wahhab MA, El-Kady AA, Hassan AM, Abd El-Moneim OM, Abdel-Aziem SH (2015) Effectiveness of activated carbon and Egyptian montmorillonite in the protection against deoxynivalenol-induced cytotoxicity and genotoxicity in rats. Food and Chemical Toxicology 83:174-182 doi:10.1016/j.fct.2015.06.015

Andretta I, Kipper M, Lehnen C, Hauschild L, Vale M, Lovatto P (2012) Meta-analytical study of productive and nutritional interactions of mycotoxins in growing pigs. Animal 6(9):1476-1482

Azizi T, Daneshyar M, Allymehr M, Jalali AS, Behroozyar HK, Tukmechi A (2021) The impact of deoxynivalenol contaminated diet on performance, immune response, intestine morphology and jejunal gene expression in broiler chicken. Toxicon

Bolliger AP, Everds N, Zimmerman K, Moore D, Smith S, Barnhart K (2010) Hematology of laboratory animals. Schalm’s Veterinary Hematology 6:852-862

Bracarense APF, Basso KM, Da Silva EO, Payros D, Oswald IP (2017) Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. World Mycotoxin Journal 10(1):89-96 doi:10.3920/WMJ2016.2094

Brera C, de Santis B, Debegnach F, et al. (2015) Experimental study of deoxynivalenol biomarkers in urine. EFSA Supporting Publications 12(6):818E

Chattopadhyay P, Upadhyay A, Agnihotri A, Karmakar S, Ghoyary D, Veer V (2013) Comparative hematoxicity of fusirium mycotoxin in experimental sprague-dawley rats. Toxicol Int 20(1):25

Clark ES, Flannery BM, Pestka JJ (2015) Murine Anorectic Response to Deoxynivalenol (Vomitoxin) Is Sex-Dependent. Toxins 7(8):2845-2859

Conforto TL, Waxman DJ (2012) Sex-specific mouse liver gene expression: genome-wide analysis of developmental changes from pre-pubertal period to young adulthood. Biology of sex differences 3(1):1-16 doi:10.1186/2042-6410-3-9

Cote LM, Beasley VR, Bratich PM, Swanson SP, Shivaprasad HL, Buck WB (1985) Sex-Related Reduced Weight Gains in Growing Swine Fed Diets Containing Deoxynivalenol. Journal of Animal Science 61(4):942-950 doi:10.2527/jas1985.614942x

Da Silva E, Bracarense AP, Oswald IP (2018) Mycotoxins and oxidative stress: where are we? World Mycotoxin Journal 11(1):113-134 doi:10.3920/WMJ2017.2267

Da Silva EO, Gerez JR, Hohmann MS, Verri WA, Bracarense APF (2019) Phytic acid decreases oxidative stress and intestinal lesions induced by fumonisin B1 and deoxynivalenol in intestinal explants of pigs. Toxins 11(1):18 doi:10.3390/toxins11010018

Eriksen GS, Pettersson H (2004) Toxicological evaluation of trichothecenes in animal feed. Animal Feed Science and Technology 114(1):205-239 doi:https://doi.org/10.1016/j.anifeedsci.2003.08.008

Fattori V, Pinho-Ribeiro FA, Borghi SM, et al. (2015) Curcumin inhibits superoxide anion-induced pain-like behavior and leukocyte recruitment by increasing Nrf2 expression and reducing NF-κB activation. Inflammation research : official journal of the European Histamine Research Society [et al] 64(12):993-1003 doi:10.1007/s00011-015-0885-y

Feldman BF, Zinkl JG, Jain NC (2000) Schalm's veterinary hematology,

Ghareeb K, Awad W, Zebeli Q, Böhm J (2016) Deoxynivalenol in chicken feed alters the vaccinal immune response and clinical biochemical serum parameters but not the intestinal and carcass characteristics. Journal of animal physiology and animal nutrition 100(1):53-60

Greene DM, Azcona-Olivera JI, Murtha JM, Pestka JJ (1995) Effects of dihydrotestosterone and estradiol on experimental IgA nephropathy induced by vomitoxin. Toxicological Sciences 26(1):107-116

Greene DM, Azcona-Olivera JI, Pestka JJ (1994a) Vomitoxin (deoxynivalenol)-induced IgA nephropathy in the B6C3F1 mouse: dose response and male predilection. Toxicology 92(1-3):245-260

Greene DM, Bondy GS, Azcona-Olivera JI, Pestka JJ (1994b) Role of gender and strain in vomitoxin-induced dysregulation of IgA production and IgA nephropathy in the mouse. Journal of Toxicology and Environmental Health, Part A Current Issues 43(1):37-50

Grenier B, Applegate TJ (2013) Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals. Toxins (Basel) 5(2):396-430 doi:10.3390/toxins5020396

He Q, Su G, Liu K, et al. (2017) Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the nonparametric rank percentile method. PloS one 12(12):e0189837

Hou Y-J, Zhao Y-Y, Xiong B, et al. (2013) Mycotoxin-containing diet causes oxidative stress in the mouse. PloS one 8(3):e60374

Ihedioha JI, Noel-Uneke OA, Ihedioha TE (2013) Reference values for the serum lipid profile of albino rats (Rattus norvegicus) of varied ages and sexes. Comparative Clinical Pathology 22(1):93-99

Iverson F, Armstrong C, Nera E, et al. (1995) Chronic feeding study of deoxynivalenol in B6C3F1 male and female mice. Teratogenesis, carcinogenesis, and mutagenesis 15(6):283-306 doi:10.1002/(sici)1520-6866(1996)15:6<283::aid-tcm5>3.0.co;2-e

JECFA (2001) Evaluation of certain mycotoxins in food: fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization

JECFA (2011) Safety evaluation of certain contaminants in food: prepared by the Seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Kachlek M, Szabó-Fodor J, Szabó A, et al. (2017) Subchronic exposure to deoxynivalenol exerts slight effect on the immune system and liver morphology of growing rabbits. Acta Veterinaria Brno 86(1):37-44

Kaneko JJ, Harvey JW, Bruss ML (2008) Clinical biochemistry of domestic animals. Academic press

Katalinic V, Modun D, Music I, Boban M (2005) Gender differences in antioxidant capacity of rat tissues determined by 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. Comparative biochemistry and physiology Toxicology & pharmacology : CBP 140(1):47-52 doi:10.1016/j.cca.2005.01.005

Kouadio JH, Dano SD, Moukha S, Mobio TA, Creppy EE (2007) Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon 49(3):306-317 doi:https://doi.org/10.1016/j.toxicon.2006.09.029

Kouadio JH, Mobio TA, Baudrimont I, Moukha S, Dano SD, Creppy EE (2005) Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. Toxicology 213(1-2):56-65 doi:10.1016/j.tox.2005.05.010

Kouadio JH, Moukha S, Brou K, Gnakri D (2013) Lipid metabolism disorders, lymphocytes cells death, and renal toxicity induced by very low levels of deoxynivalenol and fumonisin b1 alone or in combination following 7 days oral administration to mice. Toxicol Int 20(3):218-223 doi:10.4103/0971-6580.121673

Laffan SB, Posobiec LM, Uhl JE, Vidal JD (2018) Species Comparison of Postnatal Development of the Female Reproductive System. Birth defects research 110(3):163-189 doi:10.1002/bdr2.1132

Lattanzio VMT, Solfrizzo M, De Girolamo A, Chulze SN, Torres AM, Visconti A (2011) LC–MS/MS characterization of the urinary excretion profile of the mycotoxin deoxynivalenol in human and rat. Journal of Chromatography B 879(11):707-715 doi:10.1016/j.jchromb.2011.01.029

Liang Z, Ren Z, Gao S, et al. (2015) Individual and combined effects of deoxynivalenol and zearalenone on mouse kidney. Environmental Toxicology and Pharmacology 40(3):686-691 doi:https://doi.org/10.1016/j.etap.2015.08.029

Luo S, Terciolo C, Bracarense A, Payros D, Pinton P, Oswald IP (2019) In vitro and in vivo effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in a mixture on the intestinal barrier. Environment international 132:105082 doi:10.1016/j.envint.2019.105082

Manchope MF, Artero NA, Fattori V, et al. (2018) Naringenin mitigates titanium dioxide (TiO(2))-induced chronic arthritis in mice: role of oxidative stress, cytokines, and NFκB. Inflammation research : official journal of the European Histamine Research Society [et al] 67(11-12):997-1012 doi:10.1007/s00011-018-1195-y

Mishra S, Dwivedi PD, Pandey HP, Das M (2014) Role of oxidative stress in Deoxynivalenol induced toxicity. Food and Chemical Toxicology 72:20-29 doi:https://doi.org/10.1016/j.fct.2014.06.027

Mishra S, Srivastava S, Dewangan J, Divakar A, Kumar Rath S (2019) Global occurrence of deoxynivalenol in food commodities and exposure risk assessment in humans in the last decade: a survey. Critical Reviews in Food Science and Nutrition:1-29 doi:10.1080/10408398.2019.1571479

Papageorgiou M, Wells L, Williams C, et al. (2018a) Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents. Toxins (Basel) 10(2) doi:10.3390/toxins10020050

Papageorgiou M, Wells L, Williams C, et al. (2018b) Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents. Toxins 10(2):50

Pestka JJ (2010a) Deoxynivalenol-induced proinflammatory gene expression: mechanisms and pathological sequelae. Toxins 2(6):1300-1317

Pestka JJ (2010b) Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Archives of Toxicology 84(9):663-679 doi:10.1007/s00204-010-0579-8

Pestka JJ, Amuzie CJ (2008) Tissue distribution and proinflammatory cytokine gene expression following acute oral exposure to deoxynivalenol: comparison of weanling and adult mice. Food and Chemical Toxicology 46(8):2826-2831

Pestka JJ, Clark ES, Schwartz-Zimmermann HE, Berthiller F (2017) Sex Is a Determinant for Deoxynivalenol Metabolism and Elimination in the Mouse. Toxins 9(8):240 doi:10.3390/toxins9080240

Pierron A, Bracarense APF, Cossalter A-M, et al. (2018) Deepoxy-deoxynivalenol retains some immune-modulatory properties of the parent molecule deoxynivalenol in piglets. Archives of toxicology 92(11):3381-3389

Sahu SC, O'Donnell MW, Jr., Wiesenfeld PL (2010) Comparative hepatotoxicity of deoxynivalenol in rat, mouse and human liver cells in culture. Journal of applied toxicology : JAT 30(6):566-73 doi:10.1002/jat.1527

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical biochemistry 25(1):192-205 doi:10.1016/0003-2697(68)90092-4

Shifrin VI, Anderson P (1999) Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. Journal of biological chemistry 274(20):13985-13992

Silva MV, Pante GC, Romoli JCZ, et al. (2018a) Occurrence and risk assessment of population exposed to deoxynivalenol in foods derived from wheat flour in Brazil. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment 35(3):546-554 doi:10.1080/19440049.2017.1411613

Silva MV, Pante GC, Romoli JCZ, et al. (2018b) Occurrence and risk assessment of population exposed to deoxynivalenol in foods derived from wheat flour in Brazil. Food Additives & Contaminants: Part A 35(3):546-554 doi:10.1080/19440049.2017.1411613

Soler L, Oswald IP (2018) The importance of accounting for sex in the search of proteomic signatures of mycotoxin exposure. Journal of Proteomics 178:114-122 doi:https://doi.org/10.1016/j.jprot.2017.12.017

Szabó A, Szabó-Fodor J, Fébel H, et al. (2018) Individual and combined effects of fumonisin b1, deoxynivalenol and zearalenone on the hepatic and renal membrane lipid integrity of rats. Toxins 10(1):4

Szkudelska K, Szkudelski T, Nogowski L (2002) Short-time deoxynivalenol treatment induces metabolic disturbances in the rat. Toxicology Letters 136(1):25-31 doi:https://doi.org/10.1016/S0378-4274(02)00281-3

Terciolo C, Bracarense AP, Souto PC, et al. (2019) Fumonisins at doses below EU regulatory limits induce histological alterations in piglets. Toxins 11(9):548

Ueno Y, Nakajima M, Sakai K, Ishii K, Sato N, Shimada N (1973) Comparative toxicology of trichothec mycotoxins: Inhibition of protein synthesis in animal cells. Journal of Biochemistry 74(2):285-296

Vidal A, Claeys L, Mengelers M, et al. (2018) Humans significantly metabolize and excrete the mycotoxin deoxynivalenol and its modified form deoxynivalenol-3-glucoside within 24 hours. Scientific Reports 8(1):5255 doi:10.1038/s41598-018-23526-9

Wu Q-H, Wang X, Yang W, et al. (2014) Oxidative stress-mediated cytotoxicity and metabolism of T-2 toxin and deoxynivalenol in animals and humans: an update. Archives of Toxicology 88(7):1309-1326 doi:10.1007/s00204-014-1280-0

Wu X, Kohut M, Cunnick J, Bailey T, Hendrich S (2009) Deoxynivalenol suppresses circulating and splenic leukocyte subpopulations in BALB/c mice: dose response, time course and sex differences. Food Additives & Contaminants: Part A 26(7):1070-1080 doi:10.1080/02652030902832959

Zha A, Cui Z, Qi M, et al. (2020) Dietary baicalin zinc supplementation alleviates oxidative stress and enhances nutrition absorption in deoxynivalenol challenged pigs. Current Drug Metabolism 21(8):614-625 doi:10.2174/1389200221666200302124102

5. CONCLUSÃO

No primeiro artigo **“Ovarian toxicity by fusariotoxins in pigs: Does it imply in oxidative stress?”** demonstramos que a exposição a DON e/ou FB_1 induz alterações morfológicas nos ovários de suínos, incluindo a proliferação celular e apoptose. A modulação da resposta ao estresse oxidativo foi também identificada, o que pode afetar o processo de ovulação. Esses dados demostram pela primeira vez os efeitos individual e combinado destas micotoxinas sobre a resposta ao estresse oxidativo em tecido ovariano de suínos. Considerando a similaridade histofisiológica entre suínos e ovários humanos, futuro estudos devem investigar o possível papel de micotoxinas sobre a infertilidade feminina.

No segundo artigo “**Exposure to deoxynivalenol at peripuberty alters male reproductive parameters of pubertal rats”**, nossos resultados indicam que a exposição juvenil e peripuberal de ratos ao DON pode alterar a produção de espermatozoides em testículos. DON pode entrar nos túbulos seminíferos, agindo sobre células germinativas provavelmente através de duas maneiras: 1. Por alterar a barreira hematotesticular (desde que há uma redução no número de células de Sertoli e Leydig); 2. Por espermatócitos localizados no compartimento basal. Então, a dinâmica da espermatogênese é alterada com uma aceleração dos estágios seminíferos. Esses dados demostram pela primeira vez a ação desta micotoxina em testículos de animais durante o periodo juvenil e peripuberal. Considerando que a estimativa do consumo alimentar de DON por crianças e adolescentes pode exceder a ingestão diária tolerável máxima estabelecida por agências governamentais, estudos futuros sobre os efeitos de DON em pacientes com distúrbios da puberdade são fortemente encorajados.

No terceiro artigo “**Exposure to deoxynivalenol during peripubertal periods induces ovarian damage in immature rats”** demonstramos que a ativação de vias de sinalização apoptótica em ovários de ratas pode ser um importante mecanismo na toxicidade reprodutiva de DON. Esses dados demostram pela primeira vez a ação desta micotoxina em ovários de animais alimentados com uma dieta contaminada com DON durante o período juvenil e peripuberal. Considerando que a estimativa do consumo alimentar de DON por crianças e adolescentes pode exceder a ingestão diária tolerável máxima estabelecida por agências governamentais, estudos futuros sobre os efeitos de DON em pacientes com distúrbios da puberdade são fortemente encorajados.

No quarto artigo “**Animal performance and clinical response to deoxynivalenol is sex-dependent in peripubertal rats”** nos confirmamos a predileção de machos as alterações induzidas por DON no consumo alimentar, ganho de peso e peso corpóreo. Além disso, reflexos da ação anorexígena de DON pode interferir em parâmetros bioquímicos (creatinina e triglicerídeos) de fêmeas. Em adição, sexo não é um fator determinante na toxicidade sistêmica de DON identificada por alterações hematológicas e histológicas (intestino, fígado e rim). Além disso, o estresse oxidativo parece como um importante mecanismo na nefrotoxicidade de DON. Esses dados demostram pela primeira vez a ação sistêmica desta micotoxina em animais durante o período juvenil e peripuberal. Considerando que a estimativa do consumo alimentar de DON por crianças e adolescentes pode exceder a ingestão diária tolerável máxima estabelecida por agências governamentais, este estudo traz importantes informações para agências responsáveis pela elaboração de normais de segurança alimentar.

6. REFERÊNCIAS

Abdel-Wahhab, M. A., A. A. El-Nekeety, A. S. Salman, S. H. Abdel-Aziem, F. M. Mehaya and N. S. Hassan (2018). Protective capabilities of silymarin and inulin nanoparticles against hepatic oxidative stress, genotoxicity and cytotoxicity of Deoxynivalenol in rats. Toxicon 142: 1-13.

Alm, H., T. Greising, K. P. Brüssow, H. Torner and U. Tiemann (2002). The influence of the mycotoxins deoxynivalenol and zearalenol on in vitro maturation of pig oocytes and in vitro culture of pig zygotes. Toxicol In Vitro 16(6): 643-648.

Bai, Y., K. Ma, J. Li, J. Li, C. Bi and A. Shan (2021). Deoxynivalenol exposure induces liver damage in mice: Inflammation and immune responses, oxidative stress, and protective effects of Lactobacillus rhamnosus GG. Food Chem Toxicol 156: 112514.

Bouhet, S. and I. P. Oswald (2005). The effects of mycotoxins, fungal food contaminants, on the intestinal epithelial cell-derived innate immune response. Vet Immunol Immunopathol 108(1-2): 199-209.

Cao, Z., W. Huang, Y. Sun and Y. Li (2020). Deoxynivalenol induced spermatogenesis disorder by blood-testis barrier disruption associated with testosterone deficiency and inflammation in mice. Environmental Pollution 264: 114748.

Chuffa, L. G., M. S. Alves, M. Martinez, I. C. Camargo, P. F. Pinheiro, R. F. Domeniconi, L. A. Júnior and F. E. Martinez (2016). Apoptosis is triggered by melatonin in an in vivo model of ovarian carcinoma. Endocr Relat Cancer 23(2): 65-76.

Clark, E. S., B. M. Flannery and J. J. Pestka (2015). Murine anorectic response to deoxynivalenol (vomitoxin) is sex-dependent. Toxins 7(8): 2845-2859.

Cortinovis, C., F. Pizzo, L. J. Spicer and F. Caloni (2013). Fusarium mycotoxins: Effects on reproductive function in domestic animals—A review. Theriogenology 80(6): 557-564.

Cote, L., V. R. Beasley, P. Bratich, S. Swanson, H. Shivaprasad and W. Buck (1985). Sex-related reduced weight gains in growing swine fed diets containing deoxynivalenol. Journal of animal science 61(4): 942-950.

Crocker, J. and N. Skilbeck (1987). Nucleolar organiser region associated proteins in cutaneous melanotic lesions: a quantitative study. Journal of clinical pathology 40(8): 885-889.

da Silva, E. O., J. R. Gerez, T. do Carmo Drape and A. Bracarense (2014). Phytic acid decreases deoxynivalenol and fumonisin B1-induced changes on swine jejunal explants. Toxicol Rep 1: 284-292.

da Silva, E. O., J. R. Gerez, M. S. N. Hohmann, W. A. Verri, Jr. and A. Bracarense (2019). Phytic Acid Decreases Oxidative Stress and Intestinal Lesions Induced by Fumonisin B_1 and Deoxynivalenol in Intestinal Explants of Pigs. Toxins (Basel) 11(1).

Demaegdt, H., B. Daminet, A. Evrard, M.-L. Scippo, M. Muller, L. Pussemier, A. Callebaut and K. Vandermeiren (2016). Endocrine activity of mycotoxins and mycotoxin mixtures. Food and Chemical Toxicology 96: 107-116.

Dutra Gonçalves, G., N. Antunes Vieira, H. Rodrigues Vieira, A. Dias Valério, G. Elóisa Munhoz de Lion Siervo, P. Fernanda Felipe Pinheiro, F. Eduardo Martinez, F. Alessandra Guarnier, G. Rampazzo Teixeira and G. Scantamburlo Alves Fernandes (2017). Role of resistance physical exercise in preventing testicular damage caused by chronic ethanol consumption in UChB rats. Microsc Res Tech 80(4): 378-386.

Eriksen, G. S., H. Pettersson and T. Lundh (2004). Comparative cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. Food and Chemical Toxicology 42(4): 619-624.

Escrivá, L., G. Font and L. Manyes (2015). In vivo toxicity studies of fusarium mycotoxins in the last decade: A review. Food and Chemical Toxicology 78: 185-206.

Favareto, A. P., C. D. Fernandez, D. A. da Silva, J. A. Anselmo-Franci and G. Kempinas Wde (2011). Persistent impairment of testicular histology and sperm motility in adult rats treated with Cisplatin at peri-puberty. Basic Clin Pharmacol Toxicol 109(2): 85-96.

Fink-Grernmels, J. (1999). Mycotoxins: their implications for human and animal health. Veterinary Quarterly 21(4): 115-120.

Friend, D. W., H. L. Trenholm, P. S. Fiser, K. E. Hartin and B. K. Thompson (1986). Effect of feeding diets containing deoxynivalenol (vomitoxin) - contaminated wheat or corn on the feed consumption, weight gain, organ weight and sexual development of male and female pigs. Canadian Journal of Animal Science 66(3): 765-775.

Gerez, J. R., S. S. Desto and A. P. F. R. L. Bracarense (2017). Deoxynivalenol induces toxic effects in the ovaries of pigs: an ex vivo approach. Theriogenology 90: 94-100.

Gerez, J. R., S. S. Desto and A. P. F. R. L. Bracarense (2017). Deoxynivalenol induces toxic effects in the ovaries of pigs: An ex vivo approach. Theriogenology 90: 94-100.

Gerez, J. R., P. Pinton, P. Callu, F. Grosjean, I. P. Oswald and A. P. F. L. Bracarense (2015). Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs. Experimental and Toxicologic Pathology 67(2): 89-98.

Grenier, B., A. P. Loureiro-Bracarense, J. Lucioli, G. D. Pacheco, A. M. Cossalter, W. D. Moll, G. Schatzmayr and I. P. Oswald (2011). Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. Mol Nutr Food Res 55(5): 761-771.

Guerra, M. T., M. Sanabria, G. A. Leite, C. S. Borges, M. S. Cucielo, J. A. Anselmo-Franci, W. G. Foster and W. G. Kempinas (2017). Maternal exposure to butyl paraben impairs testicular structure and sperm quality on male rats. Environ Toxicol 32(4): 1273-1289.

Hallaj Salahipour, M., S. Hasanzadeh, H. Malekinejad, M. Razi and F. Farrokhi-Ardebili (2019). Deoxynivalenol reduces quality parameters and increases DNA damage in mice spermatozoa. Andrologia 51(5): e13238.

Han, J., Q. C. Wang, C. C. Zhu, J. Liu, Y. Zhang, X. S. Cui, N. H. Kim and S. C. Sun (2016). Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during porcine oocyte maturation. Toxicol Appl Pharmacol 300: 70-76.

Joint, F., W. E. C. o. F. Additives and W. H. Organization (2011). Safety evaluation of certain contaminants in food: prepared by the Seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), World Health Organization.

Katalinic, V., D. Modun, I. Music and M. Boban (2005). Gender differences in antioxidant capacity of rat tissues determined by 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. Comp Biochem Physiol C Toxicol Pharmacol 140(1): 47-52.

Kolesárová, A., M. Capcarová, N. Maruniaková, Z. Baková, R. Toman, S. Nath and A. V. Sirotkin (2012). Deoxynivalenol-induced animal ovarian signaling: proliferation and apoptosis. Journal of Microbiology, Biotechnology and Food Sciences 9(4): 323-332.

Kouadio, J. H., T. A. Mobio, I. Baudrimont, S. Moukha, S. D. Dano and E. E. Creppy (2005). Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. Toxicology 213(1): 56-65.

Krishnaswamy, R., S. N. Devaraj and V. V. Padma (2010). Lutein protects HT-29 cells against Deoxynivalenol-induced oxidative stress and apoptosis: Prevention of NF-κB nuclear localization and down regulation of NF-κB and Cyclo-Oxygenase–2 expression. Free radical biology and medicine 49(1): 50-60.

Lan, M., J. Han, M. H. Pan, X. Wan, Z. N. Pan and S. C. Sun (2018). Melatonin protects against defects induced by deoxynivalenol during mouse oocyte maturation. J Pineal Res 65(1): e12477.

Leblond, C. P. and Y. Clermont (1952). Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfurous acid technique. Am J Anat 90(2): 167-215.

Li, D., H. Ma, Y. Ye, C. Ji, X. Tang, D. Ouyang, J. Chen, Y. Li and Y. Ma (2014). Deoxynivalenol induces apoptosis in mouse thymic epithelial cells through mitochondria-mediated pathway. Environmental toxicology and pharmacology 38(1): 163-171.

Liang, Z., Z. Ren, S. Gao, Y. Chen, Y. Yang, D. Yang, J. Deng, Z. Zuo, Y. Wang and L. Shen (2015). Individual and combined effects of deoxynivalenol and zearalenone on mouse kidney. Environmental Toxicology and Pharmacology 40(3): 686-691.

Malekinejad, H., E. J. Schoevers, I. J. Daemen, C. Zijlstra, B. Colenbrander, J. Fink-Gremmels and B. A. Roelen (2007). Exposure of oocytes to the Fusarium toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. Biol Reprod 77(5): 840-847.

Manchope, M. F., N. A. Artero, V. Fattori, S. S. Mizokami, D. L. Pitol, J. P. M. Issa, S. Y. Fukada, T. M. Cunha, J. C. Alves-Filho, F. Q. Cunha, R. Casagrande and W. A. Verri, Jr. (2018). Naringenin mitigates titanium dioxide (TiO(2))-induced chronic arthritis in mice: role of oxidative stress, cytokines, and NFκB. Inflamm Res 67(11-12): 997-1012.

Marques-Pinto, A. and D. Carvalho (2013). Human infertility: are endocrine disruptors to blame? Endocrine connections 2(3): 15-29.

Mascarenhas, M. N., S. R. Flaxman, T. Boerma, S. Vanderpoel and G. A. Stevens (2012). National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. PLOS Medicine 9(12): e1001356.

Medvedova, M., A. Kolesarova, M. Capcarova, R. Labuda, A. V. Sirotkin, J. Kovacik and J. Bulla (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells in vitro. J Environ Sci Health B 46(3): 213-219.

Milićević, D. R., M. Škrinjar and T. Baltić (2010). Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. Toxins 2(4): 572-592.

Mishra, S., P. D. Dwivedi, H. P. Pandey and M. Das (2014). Role of oxidative stress in Deoxynivalenol induced toxicity. Food and chemical toxicology 72: 20-29.

Morrissey, R. E. and R. F. Vesonder (1985). Effect of deoxynivalenol (vomitoxin) on fertility, pregnancy, and postnatal development of Sprague-Dawley rats. Appl Environ Microbiol 49(5): 1062-1066.

Nassr, A. C., A. C. Arena, F. C. Toledo, D. Z. Bissacot, C. D. Fernandez, A. L. Spinardi-Barbisan, P. W. Pires and W. G. Kempinas (2010). Effects of gestational and lactational fenvalerate exposure on immune and reproductive systems of male rats. J Toxicol Environ Health A 73(13-14): 952-964.

Ndossi, D., C. Frizzell, N. Tremoen, C. Fæste, S. Verhaegen, E. Dahl, G. Eriksen, M. Sørlie, L. Connolly and E. Ropstad (2012). An in vitro investigation of endocrine disrupting effects of trichothecenes deoxynivalenol (DON), T-2 and HT-2 toxins. Toxicology letters 214(3): 268-278.

Ojeda, S.R., Andrews, W.W., Advis, J.P., White, S.S., 1980. Recent Advances in the Endocrinology of Puberty. Endocrinology Review. 1, 228–257.

Papageorgiou, M., L. Wells, C. Williams, K. White, B. De Santis, Y. Liu, F. Debegnach, B. Miano, G. Moretti, S. Greetham, C. Brera, S. L. Atkin, L. J. Hardie and T. Sathyapalan (2018). Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents. Toxins 10(2): 50.

Pedersen, T. and H. Peters (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. Reproduction 17(3): 555-557.

Pestka, J. J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. Animal Feed Science and Technology 137(3-4): 283-298.

Pestka, J. J. and C. J. Amuzie (2008). Tissue distribution and proinflammatory cytokine gene expression following acute oral exposure to deoxynivalenol: comparison of weanling and adult mice. Food and Chemical Toxicology 46(8): 2826-2831.

Pestka, J. J., E. S. Clark, H. E. Schwartz-Zimmermann and F. Berthiller (2017). Sex is a determinant for deoxynivalenol metabolism and elimination in the mouse. Toxins 9(8): 240.

Phoophitphong, D., S. Wangnaitham, S. Srisuwatanasagul and P. Tummaruk (2012). The use of proliferating cell nuclear antigen (PCNA) immuno-staining technique to determine number and type of follicles in the gilt ovary. Livestock science 150(1-3): 425-431.

Pomothy, J. M., O. Szabó, E. Czimmermann Á, Á. Babiczky, Á. Jerzsele and E. Pászti-Gere (2021). Investigation of the inflammatory and oxidative stress-inducing effects of deoxynivalenol and T-2 toxin exposure in non-tumorigenic human intestinal cell model. Toxicon 200: 78-86.

Ranzenigo, G., F. Caloni, F. Cremonesi, P. Y. Aad and L. J. Spicer (2008). Effects of Fusarium mycotoxins on steroid production by porcine granulosa cells. Anim Reprod Sci 107(1-2): 115-130.

Sahu, S. C., L. H. Garthoff, M. G. Robl, S. J. Chirtel, D. I. Ruggles, T. J. Flynn and T. J. Sobotka (2008). Rat liver clone-9 cells in culture as a model for screening hepatotoxic potential of food-related products: hepatotoxicity of deoxynivalenol. J Appl Toxicol 28(6): 765-772.

Sahu, S. C., M. W. O'Donnell, Jr. and P. L. Wiesenfeld (2010). Comparative hepatotoxicity of deoxynivalenol in rat, mouse and human liver cells in culture. J Appl Toxicol 30(6): 566-573.

Savard, C., P. Nogues, A. Boyer and Y. Chorfi (2016). Prevention of deoxynivalenol-and zearalenone-associated oxidative stress does not restore MA-10 Leydig cell functions. Toxicology 341: 17-27.

Schoevers, E. J., J. Fink-Gremmels, B. Colenbrander and B. A. J. Roelen (2010). Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. Theriogenology 74(6): 968-978.

Sedlak, J. and R. H. Lindsay (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25(1): 192-205.

Silva, M. V., G. C. Pante, J. C. Z. Romoli, A. P. M. de Souza, G. H. O. d. Rocha, F. D. Ferreira, A. L. R. Feijó, S. M. P. Moscardi, K. R. de Paula, E. Bando, S. B. Nerilo and M. Machinski Jr (2018). Occurrence and risk assessment of population exposed to deoxynivalenol in foods derived from wheat flour in Brazil. Food Additives & Contaminants: Part A 35(3): 546-554.

Sprando, R., J. Pestka, T. Collins, J. Rorie, M. O'donnell, D. Hinton and S. Chirtel (1999). The Effect of Vomitoxin (Deoxnivalenol) on Testicular Morphology, Testicular Spermatid Counts and Epididymal Sperm Counts in IL-6KO [B6129-IL6< tmlKopf>(IL-6 gene deficient)] and WT [B6129F2 (wild type to B6129-IL6 with an intact IL-6 gene)] mice. Food and chemical toxicology 37(11): 1073-1079.

Sprando, R. L., T. F. Collins, T. N. Black, N. Olejnik, J. I. Rorie, R. M. Eppley and D. I. Ruggles (2005). Characterization of the effect of deoxynivalenol on selected male reproductive endpoints. Food and chemical toxicology 43(4): 623-635.

Strolin, M., R. Whomsley and E. L. Baltes (2005). Differences in absorption, distribution, metabolism and excretion of xenobiotics between the paediatric and adult populations. Expert opinion on drug metabolism & toxicology 1(3): 447-471.

Sun, H., T.-T. Gong, Y.-T. Jiang, S. Zhang, Y.-H. Zhao and Q.-J. Wu (2019). Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990–2017: results from a global burden of disease study, 2017. Aging (Albany NY) 11(23): 10952-10991.

Sundheim, L., I. T. Lillegaard, C. K. Fæste, A.-L. Brantsæter, G. Brodal and G. S. Eriksen (2017). Deoxynivalenol Exposure in Norway, Risk Assessments for Different Human Age Groups. Toxins 9(2): 46.

Tang, Y., J. Li, F. Li, C. A. Hu, P. Liao, K. Tan, B. Tan, X. Xiong, G. Liu, T. Li and Y. Yin (2015). Autophagy protects intestinal epithelial cells against deoxynivalenol toxicity by alleviating oxidative stress via IKK signaling pathway. Free Radic Biol Med 89: 944-951.

Urbanek, K. A., D. E. Habrowska-Górczyńska, K. Kowalska, A. Stańczyk, K. Domińska and A. W. Piastowska-Ciesielska (2018). Deoxynivalenol as potential modulator of human steroidogenesis. Journal of Applied Toxicology 38(12): 1450-1459.

Wan, M. L. Y., P. C. Turner, V. A. Co, M. F. Wang, K. M. A. Amiri and H. El-Nezami (2019). Schisandrin A protects intestinal epithelial cells from deoxynivalenol-induced cytotoxicity, oxidative damage and inflammation. Scientific Reports 9(1): 19173.

Wu, M., H. Xiao, W. Ren, J. Yin, B. Tan, G. Liu, L. Li, C. M. Nyachoti, X. Xiong and G. Wu (2014). Therapeutic Effects of Glutamic Acid in Piglets Challenged with Deoxynivalenol. PLOS ONE 9(7): e100591.

Xu, X., G. Yan, J. Chang, P. Wang, Q. Yin, C. Liu, S. Liu, Q. Zhu and F. Lu (2020). Astilbin ameliorates deoxynivalenol-induced oxidative stress and apoptosis in intestinal porcine epithelial cells (IPEC-J2). J Appl Toxicol 40(10): 1362-1372.

Yang, J.-h., J.-h. Wang, W.-b. Guo, A.-r. Ling, A.-q. Luo, D. Liu, X.-l. Yang and Z.-h. Zhao (2019). Toxic effects and possible mechanisms of deoxynivalenol exposure on sperm and testicular damage in BALB/c mice. Journal of agricultural and food chemistry 67(8): 2289-2295.

Zhang, X., L. Jiang, C. Geng, J. Cao and L. Zhong (2009). The role of oxidative stress in deoxynivalenol-induced DNA damage in HepG2 cells. Toxicon 54(4): 513-518.

Zhou, H.-R., Z. Islam and J. J. Pestka (2005). Induction of Competing Apoptotic and Survival Signaling Pathways in the Macrophage by the Ribotoxic Trichothecene Deoxynivalenol. Toxicological Sciences 87(1): 113-122.