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Deoxynivalenol induces ovarian apoptosis in peripubertal rats

Desoxinivalenol durante período pré-puberal induz apoptose em ovários de ratas imaturas

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Highlights

DON induces expression of pro-apoptotic proteins in ovarian tissue.
DON decreases expression of anti-apoptotic proteins in ovarian tissue.
DON can impact reproductive function in female adult rats.

Abstract

Puberty, governed by the endocrine system, marks the onset of reproductive functions in animals and humans through a series of physiological and biological transformations. Although the mycotoxin DON can disrupt hormonal balance and cause reproductive abnormalities, its impact on puberty-associated reproductive changes remains understudied. Considering the increased exposure of children and adolescents to DON, our study aimed to elucidate its influence on follicular integrity and the expression of pro-apoptotic proteins (BAX and Caspase-3) and anti-apoptotic protein (BCL-2) in juvenile rat ovarian tissue. We divided ten 28-day-old prepubertal Wistar rats into two dietary groups for 28 days: a control group with a mycotoxin-free diet and a DON group with a diet containing 10 mg DON/Kg. After the experiment, ovaries

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and uterus weights were recorded, and the ovaries underwent morphometric and immunohistochemical analysis. DON exposure led to significant reductions in both ovarian and uterine weights. Although DON intake did not change the number of ovarian follicles across developmental stages, we observed an increased expression of BAX and Caspase-3 and a decreased BCL-2 expression in most follicular stages and corpora lutea. In summary, DON exposure during puberty can interfere with apoptotic processes in diverse ovarian cell populations during early adulthood.
Key words: Apoptosis. Deoxynivalenol. Follicular integrity. Ovary. Rat.

Resumo

A puberdade é um processo regulado pelo sistema endócrino em que as diversas alterações fisiológicas e biológicas são responsáveis pelo início das funções reprodutivas em animais e humanos. DON pode interferir no sistema hormonal induzindo desordens endócrinas e danos reprodutivos. Entretanto, o potencial de DON em causar danos reprodutivos no período da puberdade tem sido negligenciado. Considerando que crianças e adolescentes têm um alto risco de exposição à DON, este estudo teve como objetivo avaliar o efeito desta micotoxina durante a puberdade sobre a integridade folicular e a expressão de proteínas pró-apoptóticas (BAX e Caspase-3) e anti-apoptóticas (BCL-2) em ovários de ratos jovens. Dez ratos Wistar pré-púberes (28 dias de idade) foram utilizados. Os animais foram expostos por 28 dias às seguintes dietas: 1) controle: dieta livre de micotoxinas e 2) DON: dieta contendo 10 mg DON/Kg de alimento. Ao final, os ovários e úteros foram pesados e os ovários submetidos à análise morfométrica e imuno-histoquímica. A ingestão da dieta contaminada induziu a uma redução significativa no peso dos ovários e úteros, no entanto não houve diferença no número de folículos ovarianos em todos os estágios de desenvolvimento folicular. Um aumento significativo da expressão de BAX e Caspase-3 e uma diminuição da expressão de BCL-2 foram observados na maioria dos estágios foliculares e do corpo lúteo nos animais alimentados com DON. Em conclusão, a exposição ao DON durante o período pré-púbere induz apoptose em diferentes populações de células ovarianas e pode interferir no desenvolvimento reprodutivo de animais adultos.
Palavras-chave: Apoptose. Desoxinivalenol. Integridade folicular. Ovário. Ratos.

Introduction

Puberty marks the transition from childhood to adulthood, characterized by the emergence of secondary sexual characteristics, accelerated growth, and the beginning of reproductive functions (Maranghi & Mantovani, 2012). In rats, the progression from the juvenile or prepubertal stage (~21 days of age; no uterine fluid) to the peripubertal stage (~32 days of age, presence of uterine fluid) is pivotal for attaining puberty (Ojeda et al., 1980). The first estrous cycle signals the culmination of intricate hormonal changes occurring around the 34th to 38th postnatal day (PND) (Ojeda et al., 1980). Initially, this cycle is irregular, stabilizing and maturing by the 65th PND (Laffan et al., 2018). Consequently, the peripubertal period emerges as a significant phase susceptible to toxic agents targeting the ovaries.

Deoxynivalenol (DON), a mycotoxin produced by the Fusarium species, is a frequent contaminant in essential food crops like wheat, maize, rye, barley, oats, and other cereals worldwide, posing a potential health risk (Mishra et al., 2020). Notably, in Brazil, due to the consumption patterns of bread and pasta, females across all age brackets teenagers, adults, and the elderly are exposed to higher DON levels compared to males (Silva et al., 2018). Recent research has further identified heightened DON levels in the urine of children and adolescents relative to adults and the elderly, spotlighting the amplified exposure risks for this younger demographic (De Santis et al., 2019; Deng et al., 2018; Silva et al., 2018). Yet, the potential impacts of this mycotoxin on the biological processes transpiring during female juvenile and puberty stages remain unexamined.

In animals, acute exposure to high DON doses results in symptoms like diarrhea, vomiting, leukocytosis, and hemorrhage. Conversely, chronic exposure at lower doses is linked to anorexia, diminished weight gain, reduced feeding efficiency, and alterations in the immune, neuroendocrine, and reproductive systems (Knutsen et al., 2018). *In vitro* studies have documented direct DON effects on porcine oocytes and granulosa cells (Yu et al., 2017). This toxin impairs porcine oocyte maturation by inducing nuclear anomalies, disturbing meiotic spindle structures, escalating oxidative stress, triggering autophagy, promoting apoptosis, and instigating epigenetic modifications (Alm et al., 2002; Han et al., 2016; Lan et al., 2018; Malekinejad et al., 2007; Schoevers et al., 2010). Moreover, DON exposure in porcine granulosa cells affects cell proliferation, steroidogenesis, apoptosis, and key metabolic pathways (Medvedova et al., 2011; Ranzenigo et al., 2008; Yang et al., 2020).

Using *in vivo* models in adult rodents, a diet comprising 20 mg DON/kg of feed administered to both males and females for 60 and 15 days, respectively, yielded a pronounced dip in the fertility rate. Specifically, only 50% of mating resulted in pregnancies in the treated group, as opposed to the 80% observed in the control group (Morrissey & Vesonder, 1985). Moreover, ovarian samples from adult rats exposed to DON demonstrated decreased expression levels of growth factors like insulin I and the anti-apoptotic protein BCL-2, concurrent with elevated levels of pro-apoptotic proteins BAX and Caspase-3 (Kolesárová et al., 2012). After a week of DON consumption, female mice exhibited oocytes with considerable changes in maturation rates, meiotic spindle structures, and oxygen-reactive species levels leading to subpar fertilization outcomes (Lan et al., 2018). As such, limited *in vivo* investigations have directly explored the ovarian impact of DON (Yu et al., 2017), and no study to date has probed its effects on reproductive function during puberty.

Given the aforementioned observations, this study seeks to determine whether a DON-contaminated diet during the juvenile and peripubertal stages might inflict detrimental outcomes on follicular integrity within the ovaries of young Wistar rats. Concurrently, it aims to ascertain the role of apoptotic signaling pathways in potential DON-induced follicular toxicity.

Materials and Methods

Animals and diet

Ten 21-day-old female Wistar rats were housed under pathogen-free conditions and acclimated for seven days before experimentation. They were provided with a standard Quimtia® diet and water *ad libitum.* The environment maintained a temperature of approximately 21°C, adequate ventilation (exhaustion of air), humidity levels between 40-70%, and a 12-hour light-dark cycle. The experimental procedures received approval from our institution's Animal Use Ethics Committee (CEUA protocol nº 6986.2017.27).

Experimental design

Ten juvenile animals, aged 28 days postnatal as per Laffan et al. (2018), were randomly divided into two groups: Control group (n = 5, mycotoxin-free diet) and DON group (n = 5, diet containing 10 mg DON/kg feed). For the DON-infused diet, a 40-ppm crude extract of DON was sourced from the Laboratory of Mycology, Luiz de Queiroz College of Agriculture, Universidade de São Paulo. The blend of the standard diet and DON was then formulated at the Universidade Estadual de Londrina using a commercial feed mixer. To ensure accuracy, both diets underwent testing at the Samitec Laboratory in Santa Maria – RS/Brazil using the HPLC/MS-MS method. The control diet had DON levels below the limit established (200 µg/kg at 101% accuracy).

It is noteworthy that the selected dose of DON (10 mg/kg) reflects concentrations occasionally observed in various feeds, as detailed by Grenier and Applegate (2013). The rationale behind not opting for a real-world dose was based on evidence that rats metabolize DON to DOM-1 more efficiently than humans (Lattanzio et al., 2011). Moreover, this chosen dose aligns with past research investigating DON toxicity in murine species, specifically mentioned in studies by Bracarense et al. (2017) and Clark et al. (2015).

After 28 days of either the control or contaminated diet, the animals were euthanized using an intraperitoneal overdose of sodium pentobarbital. Euthanasia was further verified through cervical spinal cord dislocation and decapitation.

Animal performance and weights of ovary and uterus

Throughout the experiment, animals were weighed weekly. Their body weight gain and food consumption were monitored and recorded every week. Following euthanasia, both the ovary and uterus were meticulously extracted and weighed.

Follicular integrity

Ovaries were harvested and fixed in a 10% buffered formalin solution before undergoing standard histological processing. Histological sections were then stained using hematoxylin-eosin. The counting of ovarian follicles and corpus luteum was facilitated using optical microscopy. Analysis was focused on a single 4-µm-thick section, while the subsequent nine sections were skipped until the hundredth histological section was reached. Follicles were categorized into three

distinct groups based on the classification by Pedersen and Peters (1968): (1) primordial and primary follicles, identified by oocytes encircled by a singular layer of either flattened or cubic granulosa cells; (2) growing follicles, which have multiple layers of granulosa cells, and preantral follicles, characterized by multiple granulosa cell layers and a discernible outline of the follicular antrum; (3) antral follicles, recognized by a prominently developed follicular antrum, and mature follicles, where the oocyte is positioned toward the antrum's periphery and encased by the radial crown's cells. For each section, the count of every follicular category and corpus luteum was noted. Post-analysis of 10 sections for every animal, an average count for each follicle type and corpus luteum was deduced for each specimen.

Immunohistochemical analyses

Programmed cell-death biomarkers were assessed using immunohistochemical assays. 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 immunohistochemical processing, ovarian tissue slides were first deparaffinized in xylene. They were then immersed in a 0.01M sodium citrate buffer and exposed to microwave radiation (700-800W) with a pH of 6.0 for antigen retrieval. After blocking endogenous peroxidase activity, the tissue sections underwent incubation with 3% BSA for an hour to avoid nonspecific binding. The slides were then incubated in a humidified chamber at 4°C overnight, with the primary antibodies rabbit polyclonal anti-BCL-2 (1:100), polyclonal anti-BAX rabbit, and rabbit monoclonal anti-caspase-3 (1:50) (Abcam, Cambridge, UK).

Following this, the slides were washed with TBS-T buffer and then incubated with a secondary antibody, either anti-mouse IgG or anti-rabbit IgG polymer, (DakoCytomation, Carpinteria, CA, USA) at room temperature for one hour. These sections were later reacted with diaminobenzidine (Sigma–Aldrich) for five minutes and counterstained using hematoxylin.

In terms of the ovarian structures, all cells within the ovarian follicles and corpus luteum, whether follicular or luteal, were accounted for. Similarly, the counting of cells exhibiting positivity for BCL-2, BAX, and caspase-3 was done. The average percentage of immunostaining for each protein present in the ovarian follicles and corpus luteum was calculated by dividing the number of follicular or luteal cells that were positive for BCL-2, BAX, and Caspase-3 by the total cell count in each structure.

For the stromal cells and oocyte reactions, the evaluation was conducted based on the staining intensity, which was rated as either absent, weak, moderate, or strong. Whenever feasible, ten fields were examined for each section.

Statistical analysis

The data are presented 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 $p < 0.05$.

Results and Discussion

Impact of DON on reproductive organ weights

Figure 1 reveals no statistical difference in body weight, weight gain, or feed intake across the experimental groups. However, a DON-contaminated diet led to a significant reduction in ovary and uterus weight by approximately 43.23% (Figure 1D).

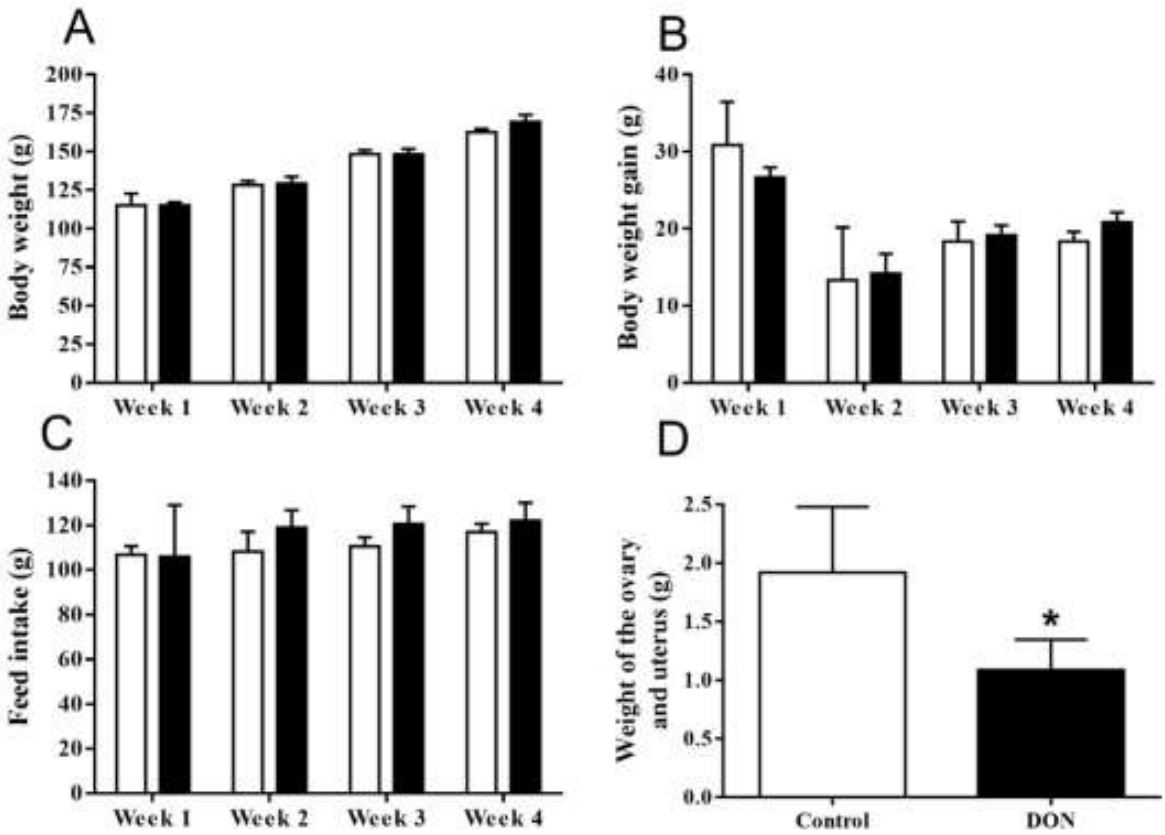

Figure 1. Effect of deoxynivalenol (DON) exposure on animal performance and weight of ovaries and uterus of peripubertal rats.
A) Weekly body weight (g - grams), B) weekly body weight gain (g), C) weekly feed intake (g), and D) weight of the animal's ovary and uterus (g). Data are presented as mean ±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

Effects of DON on follicular counting and integrity during the peripubertal period

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

Table 1
Number of 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 day 28 to 56

	Primordial and primary follicles	Growing follicles	Pre-antral follicles	Antral follicles	Mature follicles	Total follicles	Corpus luteum
Control	155.5±54.16[a]	37.00±10.22[a]	20.25±5.49[a]	13.00±4.03[a]	3.25±0.65[a]	229.5±68.64[a]	50.05±21.28[a]
DON	181.25±43.68[a]	26.00±4.60[a]	12.75±3.34[a]	9.75±3.09[a]	3.25±1.24[a]	233.25±53.71[a]	51.75±10.94[a]

Data are presented as mean ± standard deviation (n = 5). Student's t-test (p≤0.05).

Immunohistochemical analyses

In both rat groups, immunohistochemical assays assessed the protein expressions of BCL-2 (anti-apoptotic), BAX, and Caspase-3 (pro-apoptotic) within the ovaries (Figures 2 and 3).

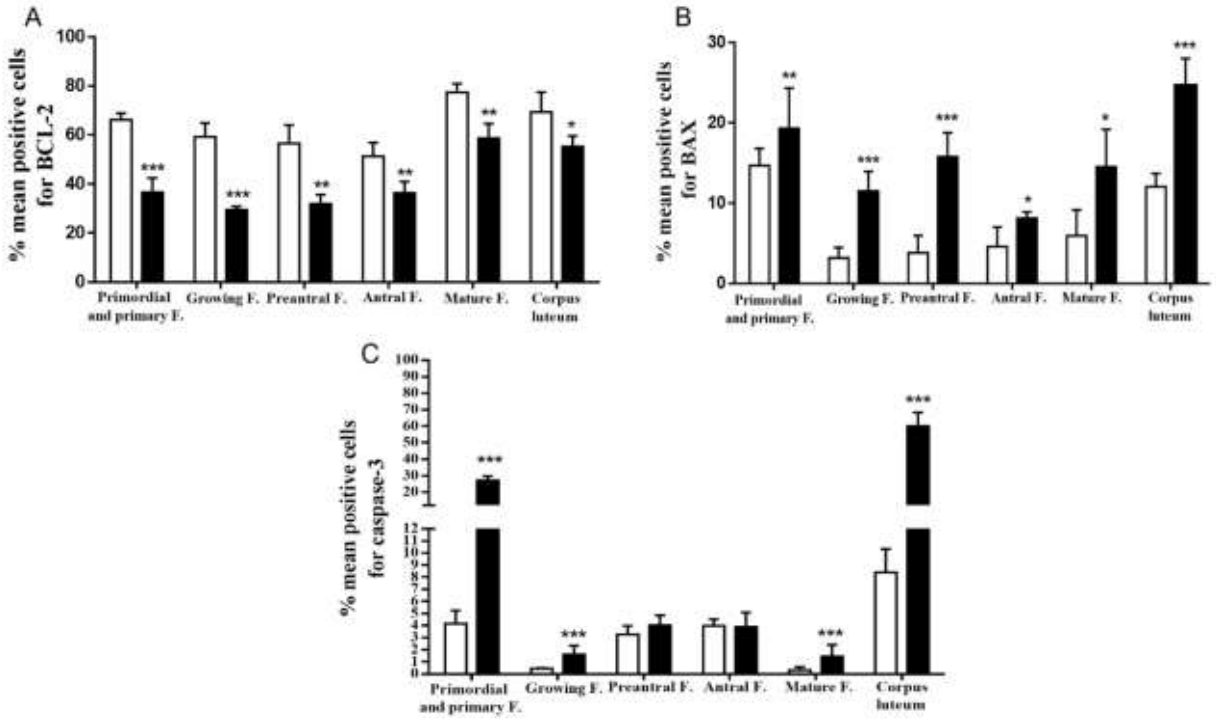

Figure 2. Effect of deoxynivalenol (DON) exposure on immune expression of BCL-2, BAX, and Caspase-3 proteins in the ovaries of young rats. Percentage of immune-positive cells for BCL-2 (A), BAX (B), and Caspase-3 (C) proteins in ovarian follicles (F.) at different developmental stages and corpus luteum 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.

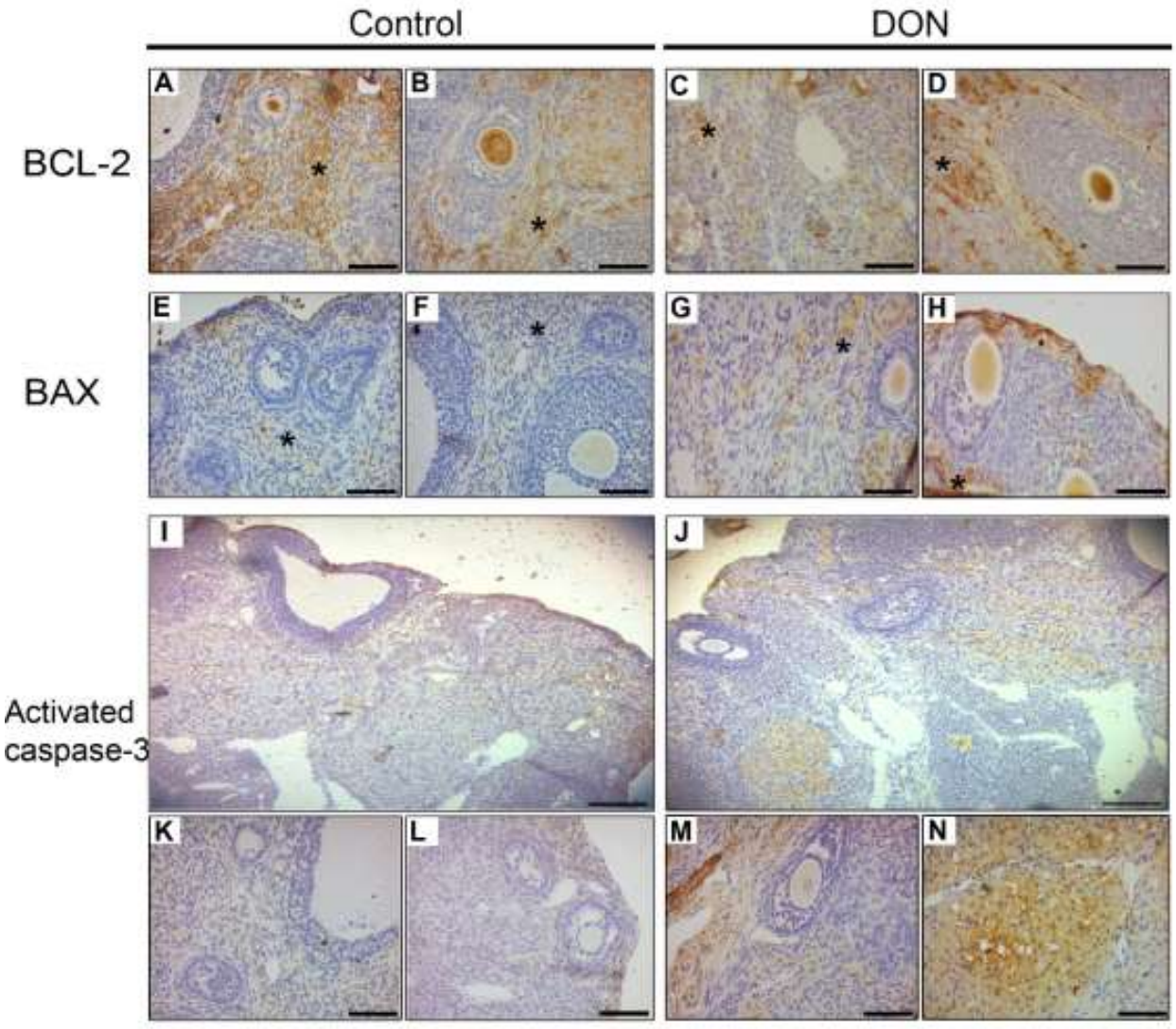

Figure 2. Effect of deoxynivalenol (DON) exposure on BCL-2, BAX, and Caspase-3 apoptosis signaling pathways in the ovaries of rats exposed to a mycotoxin-free control diet and a diet contaminated with 10 mg DON/kg during the PND 28 to 56.
(**A** and **B**) Ovaries of control animals showed strong BCL-2 immunostaining in the ovarian stroma (*), granulosa cells, and oocytes. (**C** and **D**) Ovaries of animals exposed to DON showed 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 showed moderate BAX staining in the ovarian stroma (*), oocytes, and a 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 showed 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.

For ovarian follicles, BCL-2 immunostaining significantly decreased in 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 the corpus luteum ($p=0.02$) in the DON-exposed animals compared to the control group (Figure 2A). After DON treatment, the ovarian stroma and oocyte cytoplasm exhibited a weaker to moderate immunoreaction in comparison to the pronounced immunostaining observed in the control group's ovarian stroma, indicating a decline in this anti-apoptotic protein's expression in young rat ovaries (Figure 3A-3D).

Contrastingly, the DON-exposed group displayed a surge in BAX-positive cells across all ovarian follicle stages and the corpus luteum. Specifically, there were increases of 6.98% ($p=0.009$), 8.33% ($p=0.0009$), 11.95% ($p=0.0006$), 3.51% ($p=0.03$), 8.59% ($p=0.02$), and 12.52% ($p=0.0006$) in the primordial and primary follicles, growing, preantral, antral, mature follicles, and corpus luteum compared to controls (Figure 2B). In the control group, minimal BAX protein immunostaining was noted in other ovarian structures (Figures 3E and 3F). In comparison, the DON-exposed ovaries presented moderate BAX expression in stromal cells and oocyte cytoplasm (Figure 3G-3H).

Moreover, the DON group highlighted a marked rise in Caspase-3 positive cells. The increments were 22.97% ($p<0.0001$), 1.42% ($p<0.0001$), 1.60% ($p=0.0007$), and 51.61% ($p<0.0001$) in the primordial and primary, growing, mature follicles, and corpus luteum, respectively (Figure 2C). Interestingly, Caspase-3 expression remained unchanged in preantral and antral follicles compared to controls. For stromal cells, Caspase-3 immunostaining ranged from non-existent to weak in the control group (Figure 3I, 3K, and 3L), whereas the DON-exposed group demonstrated a moderate to strong expression (Figure 3J, 3M, and 3N).

Puberty is characterized by intricate morphological, endocrine, and behavioral transformations, facilitating the transition from an immature state to reproductive maturity. In recent decades, toxicological investigations have underscored the potential impacts of certain substances on reproductive function development (Maranghi & Mantovani, 2012). Notably, studies have indicated mycotoxins as potential disruptors of puberty and fertility in both animals and humans (Eze et al., 2018; Massart et al., 2008; Massart & Saggese, 2010; R. Yang et al., 2015). Still, the repercussions of DON exposure in peripubertal animals remain elusive. Given that urinary DON levels are higher in children and adolescents than in adults (De Santis et al., 2019; Deng et al., 2018; Silva et al., 2018), exploring DON's reproductive toxicity in pubescent animals could shed light on its implications during human puberty.

In this study, we first assessed DON's impact on animal performance, focusing on ovary and uterus weights. Consistent with prior research (Andretta et al., 2012; Clark et al., 2015; Iverson et al., 1995; Rotter et al., 1994), DON exhibited no influence on female body weight, weight gain, or feed consumption. Nonetheless, animals fed the DON-contaminated diet displayed a notable decrease in ovary and uterus weights, hinting at DON's potentially toxic effects on these peripubertal organs.

The follicular integrity in pubescent ovaries remained unchanged, paralleling findings from prior studies. For instance, rat mother and pups on a DON-contaminated diet (0–2 mg kg/bw/day, corresponding to 0.3 mg DON/kg feed) for 60 days before and during pregnancy showed no ovarian histological abnormalities (Morrissey, 1984; Morrissey & Vesonder, 1985). Similarly, pigs subjected to sub-chronic or chronic consumption of DON-contaminated feed showed no notable histological differences in their ovaries (Friend et al., 1986; Trenholm et al., 1994). Contrarily, our earlier *ex vivo* studies identified compromised follicular integrity in swine ovarian explants exposed to 10 µM DON (equivalent to 3 mg/kg feed), which was characterized by a reduction in normal ovarian follicles and an increase in degenerated follicles at all follicular development stages (Gerez et al., 2021, 2017). These discrepancies might stem from differing methods (*ex vivo* versus *in vivo*) and variations in toxin exposure, underscoring the relevance of exposure mode when gauging DON's reproductive toxicity.

Ovarian follicle apoptosis during puberty critically governs the reproductive lifespan (Liew et al., 2017). Our study elucidated DON's modulation of apoptotic pathways. Specifically, DON augmented pro-apoptotic pathways (BAX and Caspase-3) while suppressing anti-apoptotic BCL-2 expression. Analogous ovarian changes due to DON had been already documented. For instance, in adult rat ovarian explants exposed to 0.034, 0.34, and 3.4 µM of DON for 24 hours, BCL-2 expression notably decreased, while expressions of BAX and Caspase-3 significantly increased (Kolesárová et al., 2012). Interestingly, Caspase-3 upregulation was not consistent throughout follicular development. DON affected both the initial stages (primordial and primary follicles) and the final stages (mature follicles and corpus luteum), while pre-antral and antral follicles were comparable to the control. The antral cavity is filled with follicular fluid (FF), produced by granulosa cells, which comprises hormones, growth factors, nutrients, and antioxidants (Da Broi et al., 2018). We hypothesize that during these developmental stages, components of the FF provide a protective mechanism, preventing cells from initiating the apoptotic process. Yet, it remains unclear why this protective effect does not persist through the final stages of follicular maturation.

Additionally, *in vitro* studies on sow granulosa cells have shown that DON, at concentrations ranging from 500 to 2000 µg/L, induces a dose-dependent escalation in apoptotic rates compared to controls (M. Yang et al., 2020). Comparable findings were noted in another study where bovine granulosa cells exposed to DON at 100 ng/mL over a span of 4 days exhibited a 15% increase in apoptotic cells (Guerrero-Netro et al., 2015). Moreover, elevated expression levels of apoptotic proteins, including Caspase-3, Caspase-9, poly (ADP-ribose) polymerase (PARP), and a heightened BAX/BCL-2 ratio, were identified in the endometrial stromal cells of mice subjected to this mycotoxin (Dai et al., 2017). DON's propensity to inhibit protein synthesis via ribosomal binding makes it a known inducer of apoptosis across diverse cell types (Lucioli et al., 2013; Pestka & Smolinski, 2005).

Moreover, while recent literature hints at a correlation between mycotoxin exposure and compromised fertility (Eze et al., 2018), *in vivo* studies detailing DON's

impact on ovaries remain limited (Yu et al., 2017). An earlier investigation involving gilts on a diet contaminated with zearalenone and DON documented an up-regulation of apoptotic caspase-3 in the ovaries and uteri and a simultaneous down-regulation of the anti-apoptotic protein Bcl-2 in the ovaries (Shi et al., 2018). As such, our current study underscores the potential role of apoptotic signaling activation in peripubertal ovaries as a pivotal mechanism in DON's reproductive toxicity in *in vivo* scenarios. Given the potential for children and adolescents' dietary DON intake to surpass the established temporary tolerable daily thresholds set by regulatory bodies (Papageorgiou et al., 2018; Sundheim et al., 2017), further exploration into DON's effects on those with puberty disorders is imperative.

Conclusions

This study pioneers the evaluation of peripubertal exposure to DON on ovarian parameters. Our findings emphasize that the activation of apoptotic pathways in murine pubertal ovaries plays a significant role in DON's reproductive toxicity. Existing data from adult animals echo a comparable trend, indicating that regardless of age, DON acts as a reproductive disruptor. Given these outcomes, we strongly advocate for additional research, encompassing functional analyses and the identification of substances that can shield animals from DON's reproductive toxicity.

Declaration of Competing Interest

The authors report no declarations of interest.

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