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Acute intoxication with single oral dose of ochratoxin A (OTA) causes leukopenia, heteropenia, lymphopenia and lymphoid depletion in the bursa of Fabricius in broiler chicks

Intoxicação aguda com dose oral única de ocratoxina A (OTA) causa leucopenia, heteropenia, linfopenia e depleção linfoide na bolsa de Fabricius em pintainhos de corte

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Highlights

Single and high oral dose of OTA was capable of causing injuries to broiler chicks.
OTA showed a tendency of reduction in anti-NDV vaccine titers transferred to chicks.
OTA affected chicks' parameters 14 days post-intoxication.

Abstract

Ochratoxin A (OTA) is a mycotoxin produced by species of Penicillium and Aspergillus, agricultural product contaminants. Chronic and sub-chronic OTA intoxication by chickens ingesting contaminated feed, leads to health damages due to its hepatotoxic, nephrotoxic, cytotoxic, immunotoxic, gastrotoxic, and possibly carcinogenic effects. As there are few data on acute intoxication, the present study evaluated the effects of a single acute OTA intoxication dose on immunological and hematological parameters in chicks. Sixteen one-day-old chicks were used, separated into two groups (n=8). A single dose of OTA (1400 µg kg-1 body weight) was administered, via gavage, for the OTA group and one dose of sterile PBS for the control group.

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On the 13th day, blood samples were collected to assess hematological and biochemical parameters, and on the 14th day, euthanasia and collection of lymphoid organs were performed. The animals of the OTA group demonstrated a significant decrease in total circulating leukocytes (p<0.001) with heteropenia (p<0.001) and lymphopenia (p=0.023), decrease hematocrit (p=0.020), hemoglobin (p=0.032), and plasma IgA (p =0.044), and increased plasma uric acid level (p=0.045), in relation to the control group. In addition, the animals intoxicated with OTA showed depletion of lymphoid cells in the bursa of Fabricius (p=0.016), but not in the thymus or spleen (p>0.05), compared to the control. For the other parameters: total plasma proteins, plasma IgY levels, and anti-Newcastle Disease Virus (NDV) vaccine titers from matrices, there were no significant differences between the analyzed groups (p>0.05), although there was a worsening tendency in contaminated animals. In conclusion, even a single acute OTA intoxication at a high dose, leads to the suppression of the systemic immune response, also affecting some hematological or biochemical parameters in chicks.
Key words: Bursa. Chicken. Leukopenia. Mycotoxin. Ochratoxin A.

Resumo

Ocratoxina A (OTA) é uma micotoxina produzida por espécies de Penicillium e Aspergillus, contaminantes de produtos agrícolas. Intoxicação crônica e subcrônica por OTA em frangos que ingerem ração contaminada, levam à danos à saúde devido aos seus efeitos hepatotóxicos, nefrotóxicos, citotóxicos, imunotóxicos, gastrotóxicos e possivelmente carcinogênicos. Como há poucos dados sobre intoxicação aguda, o presente estudo avaliou os efeitos de uma dose única de intoxicação aguda por OTA sobre parâmetros imunológicos e hematológicos em pintainhos. Foram utilizados 16 pintainhos de um dia de idade, separados em dois grupos (n=8). Uma dose única de OTA (1400 µg kg-1 de peso corporal) foi administrada, via gavagem, para o grupo OTA e uma dose de PBS estéril para o grupo controle. No 13º dia foram coletadas amostras de sangue para avaliação dos parâmetros hematológicos e bioquímicos, e no 14º dia foi realizada a eutanásia e coleta de órgãos linfoides. Os animais do grupo OTA demonstraram diminuição significativa do total de leucócitos circulantes (p<0,001) com heteropenia (p<0,001) e linfopenia (p=0,023), diminuição do hematócrito (p=0,020), hemoglobina (p=0,032) e IgA plasmática (p=0,044) e aumento do nível plasmático de ácido úrico (p=0,045), em relação ao grupo controle. Além disso, os animais intoxicados com OTA apresentaram depleção de células linfóides na bolsa de Fabricius (p=0,016), mas não no timo ou baço (p>0,05), em relação ao controle. Para os demais parâmetros: proteínas totais do plasma, níveis plasmáticos de IgY e títulos de vacinas contra o Vírus da Doença de Newcastle (NDV) das matrizes, não houve diferenças significativas entre os grupos analisados (p>0,05), embora tenha havido uma tendência de piora nos animais contaminados. Em conclusão, mesmo uma intoxicação única aguda por OTA em alta dose, leva à supressão da resposta imune sistêmica, afetando também alguns parâmetros hematológicos ou bioquímicos em pintainhos.
Palavras-chave: Bursa. Frango. Leucopenia. Micotoxina. Ocratoxina A.

Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several species of *Aspergillus* and *Penicillium*, which contaminates plant products. *Penicillium* species have an optimal growth temperature in temperate climate regions, with temperatures between 15-22 ºC, and Aspergillus in tropical climate regions, between 25-30 ºC (Malir et al., 2016; Wang et al., 2016). A variety of plant-based products are susceptible, including: corn, millet, wheat, sorghum, and soybeans, which are present as components or are important ingredients in chicken feed (Malir et al., 2016).

Different regions are susceptible to contamination, Fareed, Anjum and Ahmed (2014), in Pakistan, identified the presence of OTA in 63.15% of ingredients used in chicken feed, such as corn, rice, and soybean meal, with levels ranging from 8.8-70.7 µg kg^{-1}, as well as positivity in 29.17% of various types of ready-to-eat chicken feed, with levels between 4.4-11 µg kg^{-1}. Molina et al. (2019), in Costa Rica, identified positivity of 40.56% in cereals such as corn and wheat, with levels between 1-25 µg kg^{-1} and a positivity of 44.4% in ready-to-eat chicken feed, with mean levels between 31 µg kg^{-1}. Regarding regulation, the European Union recommends 100 µg kg^{-1} as a tolerable maximum limit of OTA in chicken feed, but in Brazil there is no regulation on maximum limits of OTA in food intended for animal consumption. It is known that when chickens ingest OTA, their products, muscles, and organs are contaminated, and this must be monitored to avoid harm to human health through the ingestion of these by-products (Iqbal et al., 2014).

In addition to the concern for human health, chronic and sub-chronic exposure of chickens to OTA affects zootechnical indices, with reduced weight gain and daily feed intake, and increased feed conversion and mortality levels. These effects are associated with the direct effects of OTA on the animal's health. Exposure to OTA causes anemia and immunosuppression by decreasing levels of immunoglobulins (IgY/IgA) and circulating leukocytes, in addition to causing depletion in lymphoid organs (Pozzo et al., 2013; Sharma et al., 2016; S. A. Khan et al., 2018; Rao et al., 2018). In biochemical parameters, it leads to decreased levels of cholesterol, triglycerides, total proteins, and albumin, increased liver injury marker enzymes, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), and injury markers of kidney enzymes, such as creatinine, uric acid, and urea, in addition to decreasing circulating Ca^{2+} and P (Jeevana et al., 2017; Pozzo et al., 2013; Rao et al., 2018). Although it is known that chronic and sub-chronic contaminations affect the performance and health of broilers, there are no studies on the effects of acute exposure to OTA that can occur during the production chain in broilers. The aim of the present study was to analyze the effects of acute contamination by OTA in chicks, using a single dose that is considered high (1400 µg kg^{-1} body weight).

Material and Methods

Mycotoxin

OTA (Cayman Chemical 111439; Ann Arbor, MI, US) was completely dissolved in absolute ethanol one day before starting the experiment, to obtain a 2000 µg mL^{-1} solution. For inoculation of the animals, the solution

was diluted in 0.5 mL of sterile phosphate buffered saline (1X PBS) at a concentration of 1400 µg kg^{-1} body weight. The concentration was chosen based on Peckham et al. (1971), in which there was mortality in one-day chickens with the use of a single dose and an OTA concentration close to that used in the present study. The authors also found 3300 µg kg^{-1} body weight of OTA as a lethal dose.

Experimental design

Sixteen-day-old chicks (*Gallus gallus, Cobb500*) were separated into two groups (n=8): control and OTA, and each group was housed in cages in separate rooms. The same feed was formulated and provided to all animals, to meet their nutritional requirements (Rostagno et al., 2011), and water was provided *ad libitum.* The animals were submitted to a photoperiod of 12 hours a day and a room temperature of 30 ºC in the first week and 28 ºC in the second week of the experiment, under strict hygiene conditions.

The experiment lasted a total of fourteen days, and on the 1st day the chicks received a dose of OTA (1400 µg kg^{-1} body weight) or a dose of sterile 1X PBS. All doses were given orally, by gavage. On the 13th day, blood was collected from the animals, and on the 14th day they were euthanized by cervical dislocation and samples of the lymphoid organs were collected (spleen, thymus, and bursa of Fabricius). The experiment was approved by the Animal Use Ethics Committee (CEUA) of the State University of Londrina, protocol number 18289.2017.16.

Hematological analysis

Whole blood was collected in tubes containing sodium heparin, and then the hematocrit was evaluated using the microhematocrit technique. The global count of nucleated cells was performed in a Neubauer chamber by means of dilution (1:200) using Natt-Herricks reagent and the differential leukocyte count was performed in the blood smear (Thrall et al., 2012). Hemoglobin was determined by the colorimetric method of cyan methemoglobin and the quantification of total plasma proteins in a refractometer (Atago Brasil Ltda., Ribeirão Preto, SP, BR).

Biochemical analysis

Blood samples from each group (n=5) were randomly chosen for biochemical analysis, and then centrifuged at 900 Xg for 10 minutes to obtain plasma. Quantification of alanine aminotransferase (ALT), urea, and uric acid was performed by the Dimension Xpand Plus integrated biochemistry system (Siemens Healthcare Diagnostics, Newark, DE, US).

Plasma antibodies

IgA/IgY Quantification was performed by an immunocapture enzyme-linked immunosorbent assay (ELISA), following the process described by Ricci et al. (2020). Plasma samples were diluted at 1:20 (IgA) and 1:250,000 (IgY) and standard serum (Bethyl Laboratories A30109; Montgomery, TX, US) diluted from 1,000 ng mL^{-1} to 15.625 ng mL^{-1} (IgA) and 200.000 ng ml^{-1} to 3.125 ng ml^{-1} (IgY).

Anti-Newcastle Disease Virus (NDV) antibody titers

To determine the anti-NDV vaccine titers from breeders, plasma samples from each group were randomly chosen (n=5), collected on the 13th day of the experiment. Direct ELISA was performed with a commercial kit to quantify anti-NDV IgY antibodies (Idexx Laboratories 99-09263; Westbrook, ME, US), according to the protocol provided by the manufacturer.

Histopathological analysis

The lymphoid organ fragments obtained (spleen, thymus, and bursa of Fabricius) were randomly chosen from each group (n=5) and fixed in a 10% buffered formalin solution. The fragments were subjected to increasing concentrations of alcohols, cleared in xylene, embedded in paraffin, and sectioned in a microtome (5 µm). Subsequently, hematoxylin/eosin staining was performed and the slides were evaluated under an optical microscope (Fischer et al., 2008). A lesion score was established to compare histological changes between groups according to a previous study (Terciolo et al., 2019).

Statistical analysis

To verify the normality and homogeneity of the two groups, the Shapiro-Wilk and Levene tests were performed, respectively. The Student's T-test was used to compare the two groups, with all data considered significantly different when $p<0.05$. In groups where there was no normality, a logarithmic statistical transformation of the data was performed, followed by the Student's T-test. The SigmaPlot 12.0 program was used to perform all statistical analyses.

Results and Discussions

OTA alters hematological parameters

The values of hematological parameters for the control and OTA groups are shown in Table 1. There were significant decreases in hematocrit ($p=0.020$) and hemoglobin levels ($p=0.032$) between the OTA and control groups. A similar result was described in chickens chronically contaminated with 56 µg kg^{-1} of OTA and 136 µg kg^{-1} of aflatoxin B1 (AFB1) in the feed (A. Khan et al., 2017). The reduction in hematocrit and hemoglobin levels is due to anemia arising from inhibition of iron absorption, cytotoxicity, and bone marrow suppression (Pozzo et al., 2013) and induction of apoptosis by the entry of Ca^{2+} through the cell membrane (Jilani et al., 2012).

Table 1
Parameters analyzed in OTA-intoxicated and non-intoxicated (control) chicks

	OTA	Control	P-value
Hematocrit (%)	27.75±1.28	30.00±2.07	**p=0.020**
Hemoglobin (g dL^{-1})	6.23±0.25	6.530±0.27	**p=0.032**
Total leucocytes (mm^3)	2035.00±281.98	3465.00±662.61	**p<0.001**
Heterophiles (mm^3)	1268.85±159.56	2170.02±413.50	**p<0.001**
Lymphocytes (mm^3)	599.50±172.21	968.27±432.56	**p=0.023**
Monocytes (mm^3)	172.70±123.46	171.60±185.90	p=0.607
Eosinophils (mm^3)	70.95±43.84	100.10±81.00	p=0.385
Basophiles (mm^3)	5.50±15.56	0±0.00	p=0.721
Total proteins (gd L^{-1})	3.05±0.18	3.17±0.22	p=0.238
ALT (U L^{-1})	2.60±2.30	0.60±0.89	p=0.108
Uric acid (mg dL^{-1})	9.14±1.51	7.36±0.78	**p=0.045**
IgA (µg mL^{-1})	2.35±1.14	4.39±3.20	**p=0.044**
IgY (mg mL^{-1})	2.10±0.59	2.74±0.83	p=0.095
Anti-NDV (titer)	597.12±220.69	838.12±560.05	p=0.316
Bursa depletion (score)	1.80±1.09	0.20±0.45	**p=0.016**

Means±standard deviation (P-value in bold when there is statistical difference). Statistics performed using the Student-t test. NDV: Newcastle Disease Virus. ALT: alanine aminotransferase. Immunoglobulin A: IgA. Immunoglobulin Y: IGY.

Regarding the leukocyte parameters (Table 1), there was a significant decrease in the number of total leukocytes in the OTA group compared to the control (p<0.001). Although the relative values did not differ between groups in the differential count (p>0.05), the animals contaminated with OTA had heteropenia (p<0.001) and lymphopenia (p=0.023) when the absolute values were observed, however, there were no differences between groups in the absolute values of eosinophils, monocytes, and basophils (p=0.385, 0.607, and 0.721, respectively).

S.A. Khan et al. (2018), found a decrease in total leukocytes with a relative increase in heterophils, eosinophils, and basophils, and a decrease in lymphocytes in chickens fed a diet containing 300 to 1100 µg kg^{-1} of OTA for 21 days. The decrease in leukocytes occurs due to the immunosuppressive action of OTA and its inflammatory action on tissues, resulting in an increase in the migration of leukocytes from typical inflammatory action, such as heterophils, eosinophils, and basophils to the tissues (Khatoon & ul Abidin, 2019). In the present study, leukopenia occurred due to heteropenia and lymphopenia, which may also indicate destruction of the hematopoietic tissue.

OTA alters plasma uric acid levels

Measurements of total plasma proteins and biochemical parameters

were performed to assess liver and kidney function. The results are shown in Table 1. The values of total plasma proteins expressed in g dL-1 of the OTA group were similar to the control group (p=0.238), and although it is not significant, it shows a decreasing trend. Other studies, using contaminated diets, found a decrease in total protein and plasma albumin levels caused by chronic OTA intoxication. This decrease is due to the loss of albumin at sites where there has been kidney damage, alterations in hepatic protein synthesis, and the mechanism of alterations in protein synthesis by inhibition of t-RNA synthase (Pozzo et al., 2013; Singh et al., 2015; Qu et al., 2017; S. A. Khan et al., 2018; Rao et al., 2018).

Regarding the renal biochemical parameter analyzed, uric acid levels in the OTA group were higher in relation to the control group (p=0.045). Other studies using contaminated diets, found an increase in plasma uric acid levels due to chronic OTA contamination (Singh et al., 2015; Rao et al., 2018), as a result of the induction of kidney damage, owing to the kidney exposure during its excretion and elimination. However, Pozzo et al. (2013), did not find an increase in uric acid levels in chickens exposed to low doses of OTA in the diet. In other words, for a significant lesion to occur with an increase in uric acid levels, a higher dose is needed.

Regarding the liver biochemical parameter analyzed, plasma ALT levels in the OTA group were similar to the control group (p=0.108). Qu et al. (2017) and Rao et al. (2018), found an increase in plasma ALT levels with chronic OTA contamination, due to the liver damage caused, with changes in the hepatocyte membrane and enzyme leakage, and to biliary hyperplasia. However, Pozzo et al. (2013), when using a low dose of OTA, within the limit recommended by the European Union for chicken feed (100 µg kg-1), also found similar levels in OTA-contaminated and non-contaminated chickens, demonstrating that it is necessary to supply OTA in higher concentrations to cause liver damage. In the current work, as a single dose, the time period between OTA inoculation and plasma collection (13 days) may have influenced the result, considering that the average half-life of ALT in birds is 47 hours (Moriles & Azer, 2021). Thus, we cannot rule out the hypothesis that initial liver damage and restoration occurred during the period, normalizing ALT levels.

OTA affects plasma IgA levels

Plasma IgA/IgY levels are shown in Table 1. Animals in the OTA group had a decrease in IgA compared to the control group (p=0.044), but similar levels of IgY (p=0.095), although with a trend to decrease. OTA is known to cause immunosuppression in animals, and studies using different concentrations of OTA have shown depletion of circulating antibody-producing immunoglobulins, leukocytes, and B lymphocytes. This is due to degeneration in the lymphoid tissues of the thymus, spleen, and bursa of Fabricius, arising from increased rates of apoptosis and inhibition of cell proliferation, with a consequent decrease in the relative weight of these organs (Pozzo et al., 2013; Sharma et al., 2016; S. A. Khan et al., 2018). The statistically similar levels of IgY may be due to the administration of a single dose, which could have been insufficient to significantly decrease these levels.

The anti-NDV IgY vaccine titers from breeders of animals in the OTA group, after 13 days, were similar in relation to the control group (p=0.316). Farooqui et al. (2019), found a decrease in vaccine antibody titers when contaminated with OTA, but used vaccinated animals during the experiment, while in the present study the antibody titers transferred from vaccinated matrices were verified for the chicks. Possibly, the decrease in vaccine titers is associated with the same immunosuppression mechanism, and in the present study, a single, high dose was not sufficient to decrease anti-NDV IgY titers. In addition, it is possible the decrease in B lymphocytes by OTA would not cause a decrease in the antibodies already present in the animals.

OTA induces lymphoid depletion in the bursa of Fabricius

Regarding the lymphoid organ injury score, no microscopic changes were observed in the spleen and thymus of animals in the OTA group or in the control group. However, a significant increase in lymphoid depletion was observed in the bursa of Fabricius in the OTA group compared to the control group (p=0.016, Figure 1). OTA is harmful to lymphoid organs, its known effects on the bursa of Fabricius are lymphoid depletion, lympholysis, accumulation of secretions, and vacuolization of cell cytoplasm, while in the spleen it causes lymphoid depletion and an increase in the number of reticulocytes in the red pulp, leading to a decrease in the relative weight of these organs (S. A. Khan et al., 2018). Although it is common for OTA poisoning to also cause degeneration in the spleen and thymus, this fact was not observed in the present study. This could be due to the type of lymphocyte population that predominates in each organ, with the bursa of Fabricius containing mainly B lymphocytes and the thymus mainly T lymphocytes, and the spleen being a secondary lymphoid organ, containing both T and B lymphocyte populations already fully mature (Kaiser & Balic, 2015).

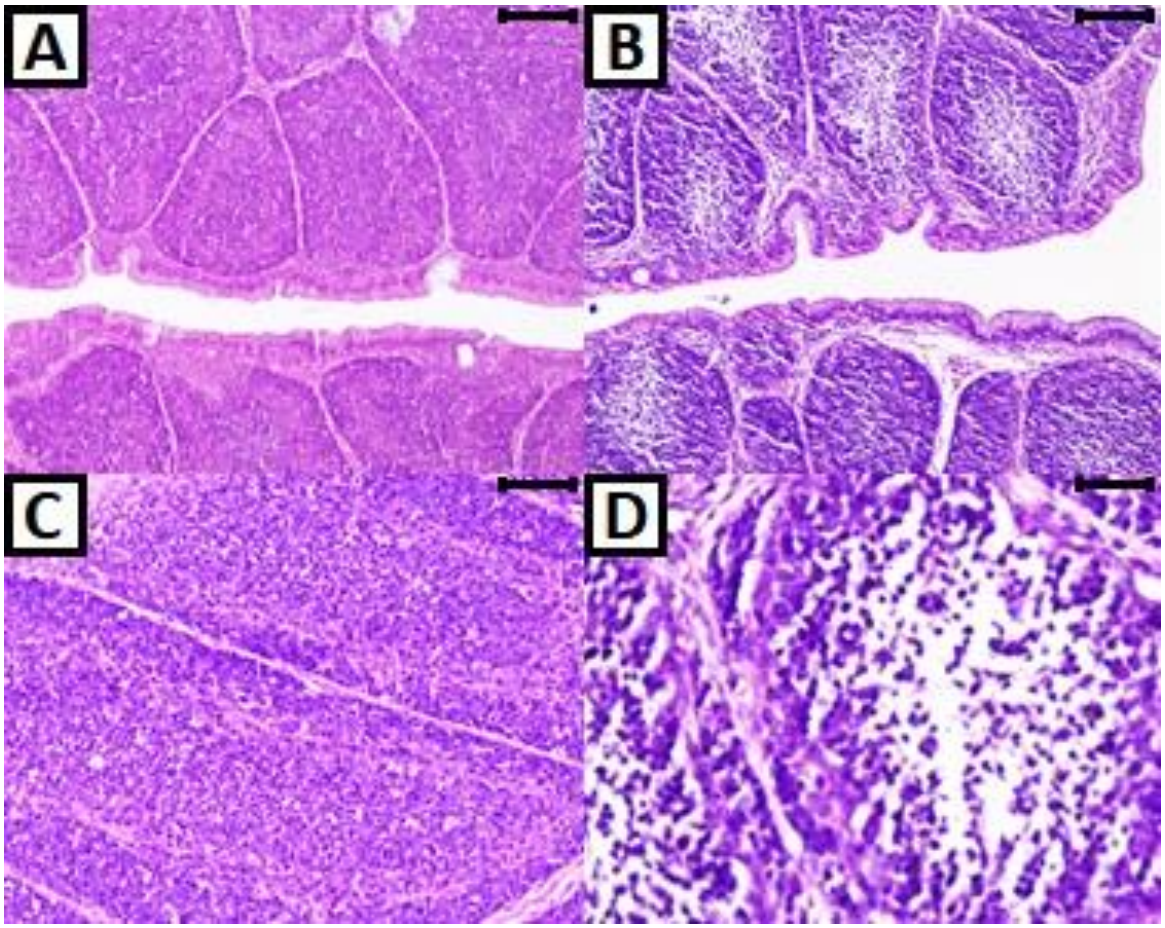

Figure 1. Bursa of Fabricius of chicks on the 14th day of the experiment, it is possible to observe lymphoid depletion in the OTA group. A: control (Bar - 100 µm). B: OTA (Bar - 100 µm). C: control (Bar - 50 µm). D: OTA (Bar - 20 µm).

Conclusions

Acute intoxication of newly hatched chicks with a single, high dose of OTA induces harmful effects on chick health, evident even 13-14 days post-exposure. The most harmful effects were leukopenia, heteropenia, and lymphoid depletion of the bursa of Fabricius. Other harmful effects such as lymphopenia, decreased serum IgA levels, and changes in uric acid levels as well as hematocrit and hemoglobin could be observed after acute OTA exposure in chicks. It is concluded that, in general, a single, high dose is harmful to chickens. This work shows the negative impact of acute intoxication, which makes it important to monitor OTA in the feed intended for use in the early life stage of chicks.

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