Toxico-pathological effects of ochratoxin A and its diastereoisomer under in ovo conditions and in vitro evaluation of the toxicity of these toxins against the embryo *Gallus gallus* fibroblast cell line

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ABSTRACT Herein, we conducted a comparative study on the embryotoxicity of ochratoxin A (**OTA**) and its diastereomer 2'R-ochratoxin A (**2'R-OTA**) under in ovo conditions, as well as assess the in vitro embryotoxicity of these substances together with ochratoxin B and α-ochratoxin, using chicken (*Gallus gallus domesticus*) embryo cell lines. In ovo tests involved egg incubation of 8 different groups (i.e., control "0"—no puncture or injection (standard incubation); "00"—punctured eggs without injection; "OTA 0.25," "OTA 0.50," "OTA 0.75," "2'R-OTA 0.25," "2'R-OTA 0.50," "2'R-OTA 0.75"—eggs containing OTA or 2'R-OTA at 0.25, 0.50, and 0.75 μg/egg concentration, respectively). The results confirmed OTA's impact on early and late embryo mortality, where chick hatchability decreased with increasing toxin dosage. Both OTA and 2'R-OTA demonstrated embryotoxicity, however, in the case of the highest OTA diastereomer dose, nearly 11% higher chick hatchability was observed compared with the group that received OTA. 2'R-OTA dosage did not reduce parameters chick quality compared to chicks hatched from control group eggs. OTA concentrations were higher than 2'R-OTA detected in chicken organs such as liver and kidney, whereas 2'R-OTA concentrations were higher in blood serum and heart. The presented studies highlighted the differences in the ability to accumulate toxins in certain organs, which, to a certain extent, may affect the potential toxicity on individual organs. Additionally, during in vitro tests, when assessing the cytotoxic effects of OTA and its analogues toward the chicken embryonic cell line in an MTT assay, the cell metabolic activity was inhibited to a comparable extent at 27-times higher concentration of 2'R-OTA than OTA (0.24 μM). Also, comparably lower toxicity was attributed to the remaining OTA derivatives.

Key words: ochratoxin A, 2'R-ochratoxin A, embryotoxicity, *Gallus gallus*

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INTRODUCTION

Ochratoxin A (**OTA**) is a mycotoxin produced by fungi from the *Aspergillus* and *Penicillium* genus, and is currently present in many agricultural products from all over the world. In warmer climates, *Aspergillus ochraceus, A. westerdijkiae*, and *A. carbonarius* biosynthesize OTA, which can infect products as such cocoa, coffee, grape juice, raisins, condiments, and many more (Magan and Aldred, 2005). In countries with a cooler climate, OTA contamination of cereals and other agricultural products (e.g., straw, hay) is associated with the growth of *Penicillium verrucosum* during storage (Magan and Aldred, 2005; Sondermann et al., 2010). Insufficiently dried grains, water condensation and insect activity

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may lead to warming up of the material and mold growth, where the growth rate depends on storage conditions and management practices (Mobashar et al., 2010).

In terms of food safety and animal welfare, the European Union has introduced legal regulations regarding the presence of mycotoxins in food and feed (Regulation 1881/2006 as amended, 2006/576/EC). In the case of OTA content in feed materials, such as cereals and cereal products as well as feeding stuffs for poultry, permissible OTA levels have been established for these products for the purpose of animal feed. The determined values are 0.25 and 0.1 mg/kg for cereals and their products and for complementary and complete feeding stuffs, respectively (2006/576/EC).

Compared with mammals, poultry show a higher sensitivity to OTA toxicity (Zhai et al., 2021). In feed used for laying hens, the presence of OTA was detected in 72% of samples, of which the majority contained OTA at 5 to 19 μg/kg (Gumus et al., 2018). Additionally, OTA presence was determined in poultry feed from some South American countries at 25 to 30 μg/kg (Magnoli et al., 2007). The main source of poultry exposure to OTA is contaminated feed. Considerably less attention has been paid to bedding studies, although it is known that contaminated cereal straw constitutes a comparable or even greater danger for bird health. Refai et al. (1996) showed that *A. ochraceus* and *P. verrucosum* produced OTA in straw at a high frequency (60% of samples), with OTA level being surprisingly high (8,000 μg/kg). Also, OTA was identified in hay and dried alfalfa samples, which is occasionally added to the bedding (Skrinjar et al., 1992; Refai et al., 1996).

Furthermore, OTA toxin can be present in the bird's tissues and eggs. Reports have confirmed the presence of OTA in meat samples (41% positive samples) with an average level of 1.41 ± 0.70 μg/kg and eggs (35%) at 1.17 ± 0.42 μg/kg (Iqbal et al., 2014). OTA content in eggs shows a consistent rise with prolonged exposure to OTA. Hassan et al. (2012a) found that OTA content in eggs reached maximum (7.4 μg/kg) after 21 d of feeding with 5 mg/kg of OTA contaminated feed. Among all known mycotoxins, OTA exhibits the highest embryotoxicity (Hassan et al., 2012b), where the increase of OTA in feed for reproductive hens, reduced the level of fertilized egg hatchability due to increased embryo mortality at various stages of development. Furthermore, OTA content was confirmed in eggs, blood plasma, liver and kidneys of adult birds and in 1-day-old chicks hatched from eggs laid by hens fed with contaminated feed (Niemiec et al., 1994). The birds' age is also important, since it is believed that chicks are more sensitive to OTA than adult birds (Adetunji et al., 2017).

According to the literature, OTA is described as a heat stable toxin. However, OTA reduction during heating at elevated temperature and the formation of its degradation products have been studied by Cramer et al. (2008). They showed that when OTA was heated at 175°C and higher, its level decreased mainly due to diastereomer formation, that is, 2'R-ochratoxin A (**2'R-OTA**). Sueck et al. (2019a) presented similar observations, where OTA isomerized at 120°C. To-date, 2'R-OTA has been found in food products that required elevated manufacturing temperatures. Although diastereomer levels were relatively low, the presence of the isomer was confirmed in coffee, cocoa, bread, and other products (Bittner et al., 2015; Bryła et al., 2021; Zapaśnik et al., 2022).

In poultry feeding, the use of granulates made from feed ingredients is very popular. Granulation is a process whereby materials with appropriate features are subjected to high pressure that generates heat. Occasionally, the addition of a heat source is used during this process. Elevated temperature in starch and lignocellulose materials leads to partial hydrolysis and dextrinization of biopolymers, which causes plasticization of the material particles (Hejft and Obidziński, 2012). However, this process improves feed digestibility, while leading to deactivation of microorganisms, which, for pathogenic bacteria, is desirable (Thomas and van der Poel, 1996). Currently, knowledge on the impact of feed pelleting on the mycotoxin transformation directions in the process is very limited. However, the possibility of OTA racemization during this process cannot be overlooked. Another potential path of poultry exposure to OTA and its diastereomer is using bedding pellet manufactured from improperly stored straw. The sole process of straw pelleting for poultry bedding purposes has various benefits from a technical, economical, and sanitary perspective, however, importantly, it is a potential source of OTA and 2'R-OTA for birds.

So far, the toxicological properties of 2'R-OTA have yet to be extensively explored. Single studies suggest that 2'R-OTA has lower toxicity than its basic form. Cramer et al. (2010) proved that the diastereomer could be characterized by an approx. Tenfold lower cytotoxic effect against human liver carcinoma cell line HepG2. Cramer et al. (2015) also detected 2'R-OTA in human blood at a comparable level to OTA. As opposed to 2'R-OTA, whose toxicological properties are poorly explored, OTA induces strong nephrotoxic, hepatotoxic, teratogenic, and immunotoxic action in poultry (Hassan et al., 2012b; Bhatti et al., 2019; Wang et al., 2019).

The presented study compared the embryotoxicity of OTA and its racemic derivative 2'R-OTA in ovo in chicken embryos, as well as an assessment of the embryotoxicity of these substances and other OTA derivatives in vitro using a chicken (*Gallus gallus domesticus*) embryo fibroblast cell line. The obtained results are the first to present comparative toxicological properties of OTA and its diastereomer and their impact on chicken embryogenesis.

MATERIALS AND METHODS

Chemicals and Reagents

The experiments employed certified OTA analytical standards (5 mg) (bought from Romer Labs (Tulln an der Donau, Austria)) and 2'R-OTA analytical standards

(10 μg/mL—bought from Aokin AG, Berlin, Germany). After purchase, OTA standard (5 mg) was quantitatively transferred to a 10 mL flask, and topped up with acetonitrile, generating OTA concentration of 500 μg/mL. Solutions of $^{13}C_{20}$—ochratoxin A (10 μg/mL), α-ochratoxin A (10 μg/mL), and ochratoxin B (10 μg/mL) analytical standards were purchased from Romer Labs. β-Glucuronidase/arylsulfatase from *Helix pomatia*, acetic acid, acetonitrile (gradient grade), hexane (LC grade), methanol (LC-MS grade), and phosphate buffered saline (**PBS**) were purchased from Merck (Darmstadt, Germany). HPLC grade methanol, propan-2-ol and acetonitrile, and 85% orthophosphoric acid were purchased from Witko Sp. z o.o. (Łódź, Poland). Deionized water, used in all experiments, was obtained from water purification plant Hydrolab (Straszyn, Poland) and Simplicity-UV purification system (Millipore, Bedford, MA). The chicken embryo fibroblasts were purchased from the American Type Culture Collection SL-29 (ATCC CRL1590) (lot number 57972861) and were cultivated in Dulbecco's modified Eagle's medium (**DMEM**, Sigma-Aldrich, Saint Louis, MO) supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) tryptose phosphate broth, L-glutamin (2 mM) and 100 units/mL penicillin and 100 mg/mL streptomycin in a CO_2 cell incubator (5% CO_2, 37°C, 98% humidity) (Binder, Germany).

2'R-OTA Synthesis for In Ovo Research and on Cell Lines

2'R-OTA was synthesized in order to obtain sufficient amount for the comparative studies of the embryotoxicity of the diastereomer. One milliliter of OTA (500 μg) solution per 5 mL was added to V-Vials, and then the solvent was evaporated from the vessel under a nitrogen stream. The vessel with OTA was placed in a KS-70S50BSS oven (Sharp Corp., Osaka, Japan) preheated to 220°C for 12 min. During this time, a racemic mixture was obtained that contained approx. 35% 2'R-OTA. Subsequently, the mixture was dissolved again in 5 mL acetonitrile. Separation of this mixture was achieved using a semipreparative liquid chromatograph by Knauer (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany), equipped with autosampler, fluorescence detector, and fraction collector. Analyte separation was conducted using a Cosmosil 5C18-AR-II Packed Column 4.6 μm × 250 mm (Nacalai Tesque, Kyoto, Japan), at 45°C. About 150 μL of the analyte solution was injected onto the column, and elution from the column was isocratic, with the phase flow rate of 1 mL/min for 40 min. The mobile phase consisted of 0.2% formic acid, acetonitrile and propan-2-ol, with a mixture ratio of 60:20:20 (v/v/v). Analyte detection was carried out at an excitation wavelength of 330 nm and emission of 460 nm. In order to obtain a single-ingredient solution containing 2'R-OTA, the fraction between 26 and 29 min retention time was collected using the collector. The analysis was repeated a total of 30 times. Analysis fractions were combined in a 200 mL round-bottom flask, then evaporated using a Hei-VAP Advantage vacuum evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 40°C. Then, the analyte was dissolved again in acetonitrile and transferred quantitatively to a 10 mL graduated flask. 2'R-OTA concentration was determined based on a calibration curve equation obtained from calibrated solutions prepared using the certified 2'R-OTA analytical standard purchased from Aokin AG (Berlin, Germany) according to a method described by Bryła et al. (2021). Then, specific volumes of 2'R-OTA solution were added to V-Vials, followed by evaporation under a nitrogen stream, followed by dissolving in 5 mL water, producing concentrations of 5, 10, and 15 μg/mL were obtained. At the same time, a control sample was prepared (pure deionized water). V-Vials were closed tightly and subjected to sonication for 10 min and autoclaved at 108°C for 3 min in order to achieve full solubility and sterility. OTA solutions with the same concentrations were in the same way prepared. Finally, 2 groups of solutions were obtained, that is, group I for 2'R-OTA: A (water), B (5 μg/mL), C (10 μg/mL), and D (15 μg/mL) and group II for OTA: A', B', C', and D' (concentrations identical as for 2'R-OTA). These solutions were used in in ovo tests of hatchable eggs.

Research Material—Eggs Collection and Experiment Design

All experimental procedures involving embryos and newly hatched chicks were approved on 2 June 2021 by the Third Local Ethics Committee on Animal Experimentation in Warsaw (Warsaw, Poland, permission No. WAW/2/078/2021).

The tests used a total of 720 hatching eggs collected at the peak of laying period (35−37 wk of life) from the Ross 308 breeder flock (Złotokłos, 05-504, PL). The hen management and feeding were in line with the Aviagen guidelines (Management Handbook, 2018). The tests were carried out in 3 reps at 7-day intervals. A total of 240 eggs from a daily collection at 35, 36, and 37 wk of life were disinfected in a water solution of Virkon S (1:100), placed on hatching trays with the blunt end facing upward and transported to the laboratory, approx. 35 km from the farm. Selected for tests were eggs without shell defects and with similar weight: 58 to 64 g.

In the laboratory, the eggs were stored at approx. 17°C and 60% RH for 24 h. Subsequently, the eggs were disinfected again using a 0.05% potassium permanganate solution ($KMnO_4$) and placed (50 s) under a UV KOR UVC-J lamp. The device used ultraviolet C (**UV-C**) range, with a radiation wavelength between 180 and 280 nm. In every repetition, the eggs were divided into 8 groups, 30 eggs each:

Control "0"—eggs incubated without puncture or injection (standard incubation)
Control "00"—eggs punctured without injection

OTA 0.25—OTA injection at 0.25 μg/egg
OTA 0.50—OTA injection at 0.50 μg/egg
OTA 0.75—OTA injection at 0.75 μg/egg
2'R-OTA 0.25—2'R-OTA injection at 0.25 μg/egg
2'R-OTA 0.50—2'R-OTA injection at 0.50 μg/egg
2'R-OTA 0.75—2'R-OTA injection at 0.75 μg/egg

The eggs were punctured with a needle 0.5 mm thick. In all groups, the toxin carrier was ultra-pure water at 50 μL/egg. After inserting the toxin, the shell puncture site was secured with a hypoallergenic plaster (Polopor by 3M Viscoplast).

Incubation was conducted under standard conditions, that is, 37.5°C to 38.5°C, 50 to 60% RH and egg position changed every 2 h by 45°, using Brinsea incubator (OvaEasy 380). The eggs were candled at 6 and 18 d of incubation. After candling at 6 d, all the eggs without living embryos were removed and broken in order to divide them into nonfertilized eggs and eggs with dead embryos—early mortality. After candling at 18 d, all the eggs with late mortality were removed, while the remaining eggs were transferred to an egg hatcher (OvaEasy). Hatching was carried out at 37.6°C and 65% RH. The chicks were weighed (±0.1 g) and quality assessment was carried out according to the methodology described by Tona et al. (2003) after modification by Damaziak et al. (2020). The chicks were assessed on a scale of 0 to 20 for activity, navel area appearance, presence of yolk sac, and leg quality, and on a scale from 0 to 5 for floccus appearance and presence of adhered egg membrane and shell fragments. The maximum score a chick could obtain was 100. All hatched chicks were killed by decapitation in order to collect blood, liver, heart, kidneys, and yolk sac for further analysis. The results obtained were used to calculate the share of early and late mortality (%), hatchability (%), body weight (g), and the share of the chick's weight in the egg weight (%), as well as the chick quality indices: the share of chicks with the full score of 100, average score for chicks in a group, the share of chicks with a score below 100.

Cytotoxicity evaluation was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt (**MTT**). Cells that were not damaged by mycotoxin, which converted the yellow tetrazolium salt, MTT, to a violet, water-insoluble formazan. The reaction occurred in the mitochondria of live cells. The intensity of color reaction was proportional to the number of intact and metabolically active cells and measured photometrically. The study was performed with the use of chicken embryo fibroblasts cell line SL-29 (ATCC CRL1590). The chicken embryo fibroblasts were cultivated in DMEM (Sigma), supplemented: tryptose phosphate broth to a final concentration of 5% and fetal bovine serum to a final concentration of 5%, L-glutamina (2 mM) and 10,000 units/mL penicillin and 10 mg/mL streptomycin. Cells were seeded at a density of 3×10^4 cells/well in 100 μL culture medium. Serial log 2 dilutions of mycotoxins sample were prepared (for OTA and 2'R-OTA: 1—17.33 μM, 2—8.67 μM, 3—4.33 μM, 4—2.17 μM, 5—1.08 μM, 6—0.54 μM, 7—0.27 μM, 8—0.14 μM, 9—0.07 μM, 10—0.03 μM; for OTB: 1—8.95 μM, 2—9.48 μM, 3—4.74 μM, 4—2.37 μM, 5—1.18 μM, 6—0.59 μM, 7—0.30 μM, 8—0.15 μM, 9—0.07 μM, 10—0.04 μM; for OTα: 1—27.28 μM, 2—13.64 μM, 3—6.82 μM, 4—3.41 μM, 5—1.71 μM, 6—0.85 μM, 7—0.43 μM, 8—0.21 μM, 9—0.11 μM, 10—0.05 μM). Test plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 48 h. Then, 20 μL MTT stock solution (reconstituted in PBS, 5 mg/mL) was added to each well, and plates were incubated for another 4 h. After incubation, MTT solution was removed and 100 μL DMSO was added to each well, and absorbance was measured spectrophotometrically using an ELISA-Reader. Cytotoxicity was quantified with a micro-plate spectrophotometer (Ledetect 96 Microplate Reader, Labexim Products, Lengau, Austria) coupled with MikroWin 2010 OEM version (Mikrotek Laborsysteme GmbH, Overath, Germany), based on absorbance measured at 510 nm wavelength, which corresponded to the maximum absorption of formazan derivative. In the case of absorbance lower than 50% of the cell division activity, all analyzed samples were considered toxic.

Sample Preparation for Mycotoxins Determination

Serum Samples About 25 μL β-glucuronidase/arylsulfatase and 375 μL PBS solution were added to 200 μL serum sample, which was mixed for 30 s and incubated overnight at 37°C. After incubation, 3.6 mL acetonitrile: water:acetic acid (89:10:1, v/v/v) mixture was added to the sample and shaken for 20 min. The mixture was centrifuged at 7,000 rpm at 6°C for 10 min and the upper layer was transferred to a 5 mL Eppendorf tube. Internal standard $^{13}C_{20}$—OTA solution (20 μL, 25.2 ng/mL) was added to the sample, and then vortexed for 30 s, followed by evaporation to dryness at 50°C under a nitrogen stream. The residue was redissolved in 200 μL 20% methanol, and then passed through a 0.20 μm Millex LC PTFE filter (Merck, Darmstadt, Germany). Finally, the supernatant was used for HPLC-MS/MS analysis.

Tissues Samples About 25 μL β-glucuronidase/arylsulfatase and 375 μL PBS solution were added to 200 mg homogenized tissue sample, and mixed for 30 s followed by incubation overnight at 37°C. Then, 3.6 mL acetonitrile:hexane:water:acetic acid (66:28:5:1, v/v/v/v) mixture was added to the sample, and shaken for 20 min. The mixture was centrifuged at 7,000 rpm at 6°C for 10 min and upper layer was transferred to a 5 mL Eppendorf tube. The mixture was centrifuged again at 10,730 × *g* at 6°C for 10 min, and the hexane phase was removed. Internal standard $^{13}C_{20}$—ochratoxin A solution (20 μL, 25.2 ng/mL) was added to the sample, and it was vortexed for 30 s, followed by evaporation to dryness at 50°C under a nitrogen stream. The residue was redissolved in 200 μL 20% methanol, and centrifuged at 7,000 rpm for 10 min. Finally, the solution was passed through a 0.20 μm Millex LC PTFE filter (Merck, Darmstadt, Germany) and the supernatant was used for HPLC-MS/MS analysis.

LC-MS/MS

HPLC-MS/MS system consisted of Shimadzu Nexera High Performance Liquid Chromatograph and 5500 QTRAP mass spectrometer (Sciex, Framingham, MA). Chromatographic separation was achieved using a Kinetex C18 column (2.1 × 100 mm, 2.6 μm, Phenomenex, Torrance, CA) at 40°C. The mobile phase was composed of 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol (B). The gradient elution program was performed as follows: 0.01−1 min, 15% B; 1−10 min, 15 to 60% B; 10−12 min, 95% B; 12−17 min, 15% B. The flow rate was 0.3 mL/min and the injection volume was 10 μL. The mass spectrometer was operated in negative mode for OTα, and positive mode for OTA, 2'R-OTA, OTB, and $^{13}C_{20}$—OTA. Quantification was achieved using multiple reaction monitoring (**MRM**) mode in one chromatographic run. The mass spectrometry was performed with electrospray ionization (**ESI**) interface at 500°C with the following settings: curtain gas: 25 psi, collision gas: medium, ionization voltage: −4,000 V (negative polarity) or +4,500 V (positive polarity), nebulizer gas: 60 psi, auxiliary gas: 60 psi. The optimization of compound dependent parameters, such as the declustering potential (**DP**), collision energy (**CE**), and collision cell exit potential (**CXP**), was performed by flow injection analysis (Table 1S Supplementary material). Analyst 1.6.2 software was used for data acquisition and processing.

Statistical Analysis

Results for continuous variables were presented as means and standard deviations; binomial variables were presented as percentages. Effect of the factors were evaluated using generalized linear model (**GLM**) with logit link function for binomial response variables and using analysis of variance (**ANOVA**) for continuous variables. Multiple comparisons of percentages were conducted using chi-squared test and multiple comparisons of means were performed using Duncan's multiple range test. The analyses were conducted in Statistica 13 program. Significance level for all the analyses was set at 0.05.

Validation Method

The method was validated by evaluating the limits of detection (**LOD**) and quantification (**LOQ**), working ranges, recoveries, precision, and expressed as repeatability. The determination of LOD and LOQ was based on a signal to noise ratio of 3 and 10, respectively, using Analyst software script. At least 6 points calibration curves were prepared for each analyte for working range determination. The recoveries were calculated by spiking mycotoxin free samples at 3 concentration levels. Three independent replicates for each concentration level were prepared to determine the precision of the method. The working ranges, LOD and LOQ for each analytes and matrix, recovery rate (R%) and method repeatability (RSD%) are shown in Tables 2S and 3S (Supplementary material).

RESULTS

The Impact of OTA and 2'R-OTA on Embryonic Development in Chickens—In Ovo Tests

The effects of OTA and 2'R-OTA injected into eggs toward hatchability and the quality of hatched chicks are shown in Table 1. Both OTA isomers, regardless of dosage, contributed to increased early and late embryo mortality and, consequently, reduced hatchability, compared with the control groups ($P < 0.05$). OTA administered dose had considerable impact on early embryo mortality, whereas 2'R-OTA impacted late mortality. The lowest hatchability was confirmed at the highest OTA dosage at as much as 11.1% ($P = 0.035$) higher than after injecting the highest dose of 2'R-OTA. No relationship between the isomer and OTA dosage was confirmed ($P > 0.05$) because early and late embryo mortality increased gradually with increasing dosage.

The toxins administered did not affect chick BW and the share of BW in the egg weight ($P > 0.05$). High OTA dosage considerably reduced the number of chicks with a score of 100, which worsened overall chick quality that was demonstrated by a very low quality index for chicks that did not achieve 100 points. 2'R-OTA injection did not reduce chick quality compared to chicks hatched from the control group's eggs.

The presence of OTA, its diastereomer, OTα and OTB that could potentially be metabolites of OTA and 2'R-OTA, was analyzed in individual isolated organs. However, the resulted showed that these metabolites were not found in any of the organs tested above the LOD. The control bird organs were also free from the tested mycotoxin metabolites. Analysis of variance demonstrated that both the substance type and the level of the tested toxins significantly impacted their accumulation in individual organs (Table 2). At all toxin dose levels used in particular groups, the highest OTA and 2'R-OTA accumulation was observed in the liver and yolk sac. OTA and 2'R-OTA content in all organs showed a significant increase with increasing dosage injected into the eggs before incubation. In the blood serum of the birds, higher concentration of 2'R-OTA (0.30−1.87 μg/kg) than OTA (0.22−0.61 μg/kg) was observed in chicks hatched from eggs injected with the same dose of OTA and 2'R-OTA. A similar tendency was found for hearts and yolk sacs. In the case of hearts, the presence of OTA and 2'R-OTA was observed only at toxin dosage of 0.50 μg/egg and 0.75 μg/egg. 2'R-OTA concentration ranged from 0.32 to 0.87 μg/kg, while OTA concentration from 0.25 to 0.59 μg/kg. In the yolk sac, 2'R-OTA concentration ranged from 4.65 to 19.83 μg/kg, while OTA concentration ranged from 7.04 to 12.40 μg/kg. A reverse tendency (i.e., a higher OTA concentration in individual organs than that of 2'R-OTA) was observed for the liver and kidneys. OTA content in the liver ranged from 4.37 to 16.02 μg/kg, while 2'R-OTA ranged from 1.11 to 12.68 μg/kg. Analogically, OTA content in kidneys ranged from 2.70 to 11.83 μg/kg, while 2'R-OTA ranged from 1.18 to 5.19 μg/kg.

Table 1. Embryo mortality, hatching, and chicks quality after in ovo injection of OTA and 2'R-OTA.

						Chicks quality		
Group	Early dead embryos, %[1]	Late dead embryos, %[2]	Hatchability, %[3]	BW (g)	BW (ratio)[4]	Chicks with score 100, %	Average score of all chicks	Average score of chicks with score <100
C	2.2%[a5]	0.0%[a]	88.9%[c]	42.55 ± 4.25[a7]	72.2 ± 5.8[a]	83.9%[bc]	97.45 ± 11.72[b]	84.20 ± 26.43[a]
C_0	4.4%[a]	0.0%[a]	81.1%[c]	42.66 ± 2.95[a]	74.1 ± 3.7[a]	80.0%[bc]	98.48 ± 4.14[b]	92.38 ± 6.52[a]
C_{00}	5.5%[a]	1.1%[a]	78.9%[c]	43.09 ± 3.37[a]	72.4 ± 4.1[a]	76.2%[bc]	95.90 ± 8.21[ab]	82.80 ± 7.43[a]
$OTA_{0.25}$	12.2%[b]	4.4%[b]	58.9%[b]	41.53 ± 3.06[a]	72.3 ± 2.9[a]	82.7%[bc]	97.02 ± 9.51[b]	82.78 ± 17.33[a]
$OTA_{0.50}$	12.2%[b]	8.9%[b]	50.0%[ab]	41.59 ± 3.12[a]	72.2 ± 2.9[a]	89.7%[bc]	98.77 ± 4.52[b]	88.00 ± 9.27[a]
$OTA_{0.75}$	31.1%[d]	4.4%[b]	35.6%[a]	41.42 ± 3.71[a]	71.6 ± 4.2[a]	63.3%[a]	91.03 ± 19.55[a]	75.55 ± 26.30[b]
$2'R\text{-}OTA_{0.25}$	14.4%[bc]	6.6%[b]	61.1%[b]	41.83 ± 2.54[a]	72.3 ± 4.0[a]	96.6%[c]	98.97 ± 5.57[b]	80.00 ± 0.00[a]
$2'R\text{-}OTA_{0.50}$	15.5%[bc]	4.4%[b]	58.9%[b]	41.59 ± 2.91[a]	72.5 ± 2.9[a]	68.6%[ab]	96.03 ± 8.37[ab]	87.36 ± 10.85[a]
$2'R\text{-}OTA_{0.75}$	21.1%[c]	11.1%[c]	46.7%[b]	42.43 ± 2.90[a]	73.1 ± 3.8[a]	70.8%[b]	94.08 ± 14.62[ab]	79.71 ± 21.88[ab]
Effect of isomer type	0.790[6]	0.575	0.161	0.318	0.214	0.991	0.633	0.713
Effect of toxin level	0.009	0.046	0.309	0.677	0.498	<0.001	0.021	0.977
Interaction between toxin type and level	0.232	0.256	0.213	0.758	0.754	0.323	0.120	0.299

C = control group − eggs without breaking the continuity of the shell and without injection; C_0 = control group in which the continuity of the shell was broken with a needle but no injection; C_{00} = control group in which the egg was injected with ultrapure water; OTA 0.25 to 0.75 = experiment in which the continuity of the shell was broken with a needle and injected with OTA at a level of 0.25, 0.50 or 0.75 μg/eggs; 2'R-OTA 0.25 to 0.75 = experiment in which the continuity of the shell was broken with a needle and injected with 2'R-OTA at a level of 0.25, 0.50, or 0.75 μg/eggs.

[1]Embryos dead up to 7 d of incubation.

[2]Embryos died after 7 d of incubation.

[3]Hatchability of fertile eggs.

[4]Chicken body weight ratio to eggs weight.

[5]Different superscript letters ($^{a-c}$) indicate significant differences at 0.05 significance level; multiple comparisons of percentages are based on chi-squared test; multiple comparisons of means are based on Duncan's multiple range test.

[6]*P* values based on generalized linear model for binomial response variables (variables presented as percentages) or based on analysis of variance (for variables presented as means). For the analyses control treatments (C, C_0, and C_{00}) were removed.

[7]Results are presented as means and standard deviations.

Table 2. Toxin levels in selected chicken tissues after in ovo injection of OTA and 2′R-OTA (in μg/kg).

Group	Serum	Liver	Heart	Kidneys	Yolk sac
C	0.00 ± 0.00a[1]	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
C_0	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
C_{00}	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
$OTA_{0.25}$	0.22 ± 0.00b	4.37 ± 0.04d	0.00 ± 0.00a	2.70 ± 0.07d	7.04 ± 0.08c
$OTA_{0.50}$	0.31 ± 0.00c	7.12 ± 0.19e	0.25 ± 0.01b	4.56 ± 0.21e	10.43 ± 0.21e
$OTA_{0.75}$	0.61 ± 0.01e	16.02 ± 0.28g	0.59 ± 0.01d	11.83 ± 0.35g	12.40 ± 0.31f
$2'R\text{-}OTA_{0.25}$	0.38 ± 0.00d	1.11 ± 0.02b	0.00 ± 0.00a	1.18 ± 0.07b	4.65 ± 0.04b
$2'R\text{-}OTA_{0.50}$	0.30 ± 0.00c	2.81 ± 0.16c	0.32 ± 0.02c	1.59 ± 0.07c	7.52 ± 0.26d
$2'R\text{-}OTA_{0.75}$	1.87 ± 0.02f	12.68 ± 0.10f	0.87 ± 0.02e	5.19 ± 0.14f	19.83 ± 0.63g
Effect of isomer type	<0.001[2]	<0.001	<0.001	<0.001	<0.001
Effect of toxin level	<0.001	<0.001	<0.001	<0.001	<0.001
Interaction between toxin type and level	<0.001	<0.001	<0.001	<0.001	<0.001

C = control group − eggs without breaking the continuity of the shell and without injection; C_0 = control group in which the continuity of the shell was broken with a needle but no injection; C_{00} = control group in which the egg was injected with ultrapure water; OTA 0.25 to 0.75 = experiment in which the continuity of the shell was broken with a needle and injected with OTA at the level of 0.25, 0.50, or 0.75 μg/eggs; 2'R-OTA 0.25 to 0.75 = experiment in which the continuity of the shell was broken with a needle and injected with 2'R-OTA at a level of 0.25, 0.50 or 0.75 μg/eggs.

[1]Results are presented as means and standard deviations. Different letters (a−g) indicate significant differences at 0.05 significance level; multiple comparisons of percentages are based on chi-squared test; multiple comparisons of means are based on Duncan's multiple range test.

[2]*P* values based on analysis of variance. For the analyses control treatments (C, C_0, and C_{00}) were removed.

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In order to determine the impact of OTA, OTB, OTα, and 2′R-OTA on chicken embryo cell viability, MTT assay was carried out using SL-29 cell line (ATCC CRL1590) exposed to increasing concentrations of the toxins for 48 h. As shown in Figure 1, exposure to all tested toxins resulted in a concentration-dependent decline in the ability to reduce tetrazolium salt to formazan by the tested cells. This effect directly resulted from the lower number of metabolically active (viable) cells. Among the toxins tested, the highest cytotoxicity was found for OTA, which inhibited the cell metabolic activity at a comparative level to OTB, OTα and 2′R-OTA, but at a 27 times lower concentration. The results were used to obtain the half maximal inhibitory concentration (**IC50**) for OTA, OTB, OTα, and 2′R-OTA (Table 3).

DISCUSSION

The Impact of OTA and 2'R-OTA on Embryonic Development in Chicks—In Ovo Tests

Poultry exposure to OTA is associated with its transfer to various organs, including breeding eggs,

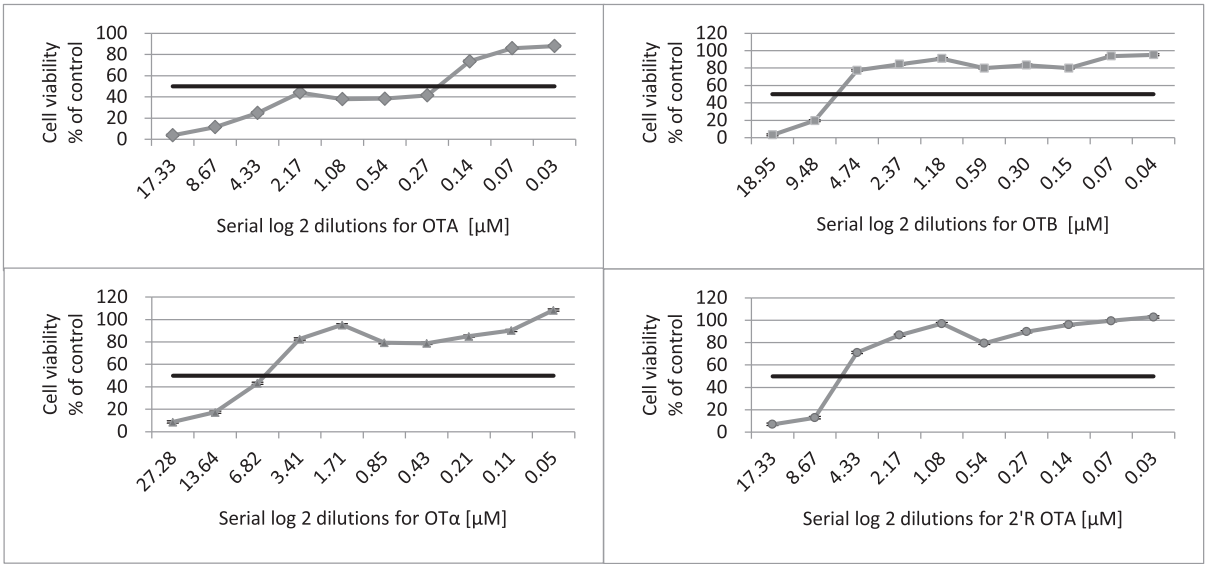

Figure 1. The effects of OTA, OTB, OTα, and 2′R-OTA on MTT reduction in SL-29 cell lines (ATCC CRL1590) after 48 h of exposure. The toxin concentrations studied were as follows: for OTA and 2'R-OTA: 1—17.33 μM, 2—8.67 μM, 3—4.33 μM, 4—2.17 μM, 5—1.08 μM, 6—0.54 μM, 7—0.27 μM, 8—0.14 μM, 9—0.07 μM, 10—0.03 μM; for OTB: 1—8.95 μM, 2—9.48 μM, 3—4.74 μM, 4—2.37 μM, 5—1.18 μM, 6—0.59 μM, 7—0.30 μM, 8—0.15 μM, 9—0.07 μM, 10—0.04 μM; for OTα: 1—27.28 μM, 2—13.64 μM, 3—6.82 μM, 4—3.41 μM, 5—1.71 μM, 6—0.85 μM, 7—0.43 μM, 8—0.21 μM, 9—0.11 μM, 10—0.05 μM.

Table 3. IC50 values for OTA, OTB, OTα, and 2′R-OTA [in μM].

Toxin	IC_{50}
OTA	0.24
OTB	7.96
OTα	5.26
2′R-OTA	6.54

contributing to, among other things, reduced reproductive indices caused by early embryo mortality, lower hatchability, and poorer conditions of hatched chicks (Hassan et al., 2010). OTA embryotoxic potential is well explored and has been confirmed in numerous studies (Malir et al., 2013; Erceg et al., 2019; Huang et al., 2019). Considering advancements in analytical techniques, new hazards have been identified (such as 2'R-OTA), which can potentially be a source of exposure in poultry. Hence, studies have been undertaken to compare the pathological impact of OTA and its diastereomer 2'R-OTA on the embryonic development and hatchability of meat chickens under in ovo conditions.

The results confirmed OTA's impact on early and late embryo mortality, with chick hatchability decreasing with increasing toxin dosage. OTA's teratogenic effects in in ovo tests at various doses have already been confirmed in previous studies by Gilani et al. (1978) as well as Vesala et al. (1983). When OTA was injected into the eggs of Leghorns at the initial phase of incubation, it was found that the minimum OTA dose leading to embryotoxicity was in the range of 0.01 to 0.05 μg. These data indicated relatively high OTA embryotoxicity. An analogical dose for a different mycotoxin, that is, citrinin, was 1 to 10 μg. Furthermore, embryos exposed to OTA demonstrated delayed embryo growth, flatulence, microphthalmia, cleft beak, deformations of extremities as well as abdominal wall and ventricular septal defects (Vesala et al., 1983). These effects were a consequence of probable production of DNA adducts and protein synthesis inhibition (Petkova-Bocharova et al., 2003). More detailed studies related to OTA's impact on chicken embryonic development were conducted by Hassan et al. (2012a), who observed the adverse impact of OTA on chicken embryonic development after 53 h incubation in terms of changes within the optical measure and the lens of the eye and body length. These changes were detected after injecting OTA at 0.01 to 1.00 μg/egg. What is more, after 9 d of incubation, teratogenic effects were observed in the embryos with increasing OTA dosage in the eggs. The most frequent embryo defect was no eye development (49%), beak hypoplasia (45%), microphthalmia (35%), beak retrognathism (12%), reduced body weight (15%), nonunited abdominal integuments (8%), cleft spine (10%), and exencephaly (4%), which were observed in the group that received OTA at 1 μg/egg. Also, embryo mortality was higher in earlier phases of development. Hatchability among fertilized eggs declined to a certain degree with increasing OTA dosage in individual groups, however, in groups with eggs containing OTA at 0.05 to 1.0 μg/egg hatchability was similar, that is, 31 to 38% (Hassan et al., 2012a). Our results showed that OTA-dose-dependent chick hatchability (0.25−0.75 μg/egg) was slightly higher, that is, 36−59%, however, this parameter was influenced by numerous factors, such as genetic factors, age, condition and nutrition of the reproductive flock and many more. The observed differences could also stemmed from the incongruity of the research models used. Although both OTA and 2'R-OTA demonstrated embryotoxicity, OTA diastereomer at the highest toxin dosage had nearly 11% higher hatchability of chicks compared with the group that received OTA. Furthermore, 2'R-OTA dosage did not reduce chick quality compared to chicks hatched from control group eggs. These results suggested lower embryotoxicity of 2'R-OTA compared with OTA. Additionally, published studies in this area lack appropriate data concerning the effects of OTA on individual embryonic developmental stages, although some reports exist related to aflatoxin B1. Neldon-Ortiz and Qureshi (1992) and Celik et al. (2000) described the maximum mortality caused by AFB1 occurring in the early developmental stages of chicken embryos.

Our obtained results did not confirm the effect of OTA and 2'R-OTA on the weight of hatched chicks, which was probably associated with the strong correlation between chick weight and egg weight. In the proposed tests, an in ovo experiment was used with eggs of similar weight in all tested groups. However, in in vivo tests, eggs from hens fed with OTA contaminated feed were characterized by lower quality associated with the thickness and strength of the shell as well as egg weight as compared with eggs from the control group fed with toxin-free feed. The higher the OTA content in the feed, the lower the egg weight (Niemiec et al., 1994). Conflicting results were obtained by Hassan et al. (2012b) in an in ovo experiment, where OTA was added to White Leghorn's eggs at dosage range of 0.01 to 1.00 μg/egg. The authors showed that the weight of hatched chicks was several percent lower than that of the control group. However, this was only evident in groups with the highest OTA dosage (Hassan et al., 2012b).

After the intake of OTA-containing feed by the animals, OTA is absorbed by passive diffusion, mainly in the jejunum, but also from the stomach (Joo et al., 2013). Due to the effect on the lipid membranes, OTA could promote degradation of its normal structure and functionality (El Cafsi et al., 2020). It enters the blood circulation through the portal vein, while the absorption rate from the gastrointestinal tract was higher in areas with lower pH. Upon uptake of OTA to the digestive tract, it had an extremely high affinity to albumin, which led to a prolonged half-life, that is, from several hours to even a month (depending on the species) (Zhai et al., 2021). In the case of chickens, the half-life was relatively shorter and amounts to 4.1 h (Galtier et al., 1981). It has already been reported that in pigs OTA accumulated in the following order of organs kidneys > liver > muscle tissue and fat (Harwig et al., 1983), with the highest concentrations found in the kidneys and

liver. However, liver was the most exposed organ as it is involved in detoxification and elimination of OTA from the blood (Joo et al., 2013). The obtained results showed that the tendency was reverse (i.e., higher toxin content in the liver than in the kidneys). This probably stemmed from the different experiment models. In in vivo tests, animals fed with OTA-containing feed, the above-mentioned organs constituted to OTA accumulation. Furthermore, after a hen eats OTA-containing feed, the toxin is transferred by the ovary to the reserve substance of the ovarian follicle and, as a consequence, to the egg yolk. In the case of our model, OTA introduced to the egg white was distributed within and partially penetrated the yolk. The white fraction was used by embryos in the final phase of egg incubation. Therefore, it was assumed that the chickens were mostly exposed to the toxin in the hatching phase and after hatching when they used the resorbed egg white and yolk reserves. The presence of OTA and 2'R-OTA in the absorbed yolk sac at a relatively high level indicated that the compounds were not fully absorbed by the developing embryo. Furthermore, it must be noted that 2'R-OTA concentrations found in the yolk sac were significantly higher than OTA concentrations for each of the 3 levels of toxin injected into the eggs. At the same time, 2'R-OTA concentrations were lower in the liver and kidneys at generally higher toxin content in the blood serum. The differences observed may suggest the existence of differences in toxin absorption, resulting from their diverse transport to yolk blood vessels and then to individual embryo organs. Unfortunately, there are no reports that describe the mechanism of OTA absorption to the blood vessels of the developing embryo. However, our observation of higher 2′R-OTA levels in blood are in good agreement with Sueck et al. (2019b) who calculated a biological half-life of approx. 6 mo in humans for 2′R-OTA compared to 1 mo for OTA.

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The presented studies demonstrated that 2'R-OTA inhibited cell metabolic activity, which was comparable to that of the other analogues except at 27-times higher OTA concentration. Therefore, 2'R-OTA had a clearly lower embryotoxic potential than OTA. Similar conclusions were drawn for the other remaining toxins. So far, only several studies related to OTA embryotoxicity in vitro using cell lines have been carried out. Although OTA embryotoxicity in different types of studies has been widely assessed, there is very limited studies for the remaining OTA derivatives. Our obtained results suggested that heat-induced OTA isomerization led to the production of OTA diastereomer that could constitute to a strategy to reduce OTA toxicity. Similar conclusions were determined by Bruinink et al. (1997), who compared the toxicity of OTA and its noncharacterized epimer induced by high temperature (according to the authors, it was 3S-epimer OTA [**3S-OTA**]). The reported study was conducted using embryonic fibroblasts of the meninges of Tetra SL laying chickens. In this case, cytotoxicity measured by mitochondrial activity, lysosomal activity and cell membrane integrity, induced through 3S-OTA and OTB, was comparable to that of OTA at 10- and 19-times higher concentration, respectively. Such significant differences in the induced toxic effects with small differences in molecular structure between OTA and 3S-OTA suggested that the isocoumarin part of ochratoxins was of key importance in this aspect (Bruinink et al., 1997). However, Cramer et al. showed that the modification was not on the dihydroisocoumarin ring but at the phenylamine moiety. Moreover, Cramer et al. (2010) could show that the in HEPG2 cells 3R- or 3S-configuration had no effect on the cytotoxicity while the 14R-derivatives were by a factor of 10 less cytotoxic. According to the literature, OTB toxicity is concordant to its considerably lower toxicity compared with OTA (Bruinink et al, 1997; Heussner et al., 2015). According to the literature, OTB toxicity is concordant to its considerably lower toxicity compared with OTA (Bruinink et al., 1997; Heussner et al., 2015). Only an in vivo study on chicks involving OTB intake per os allowed estimation of its 7-day LD50 lethal dose, which was determined as 1,890 μg/chick and corresponded to 54 mg/kg body weight. In the case of OTA, these values were 116 μg/chick, which was equivalent to 3.3 mg/kg body weight (Peckham et al., 1971). OTB (a dechlorinated form of OTA) can be produced in chickens in vivo from OTA as a result of liver microsome activity, however, the dechlorination ability is strongly dependent on the species, while the efficiency of this reaction in chickens is rather low (Yang et al., 2015). In terms of OTα, there are only a few literature reports on its toxicity. In vitro studies that examine the acute toxicity using HeLa cells concluded that OTα was much less toxic than OTA. The half maximal inhibitory concentration (IC50) amounted to 560 μM and 5 μM for OTα and OTA, respectively (Xiao et al., 1996). Clear differences in IC50 values were also obtained in cytotoxicity studies using IHKE cells. For OTA, this value was 0.5 μM, while OTα was nontoxic up to 50 μM (Bittner et al., 2015). Bruinink et al. (1998) in vitro studies used serum-free cultures of embryonic brains of Tetra SL chick stage 29, and found that OTA neurotoxic effects during 8-day low-dose (in nM) OTA exposure; such effects, however, were not observed for OTα up to 15 μM. OTA clearly affected dehydrogenase activity, lysosome activity, cell culture total protein content and neurofilament content (Bruinink et al., 1998). Furthermore, studies involving embryos of Danio rerio showed negligible teratogenicity and mortality, which only slightly differed from the results reported for negative control embryos. The same study indicated high OTA teratogenicity, which resulted in decreased growth and hatching rate, evident body deformations and mortality of some embryos (Haq et al., 2016). Although the studies cited were conducted using different types of cell lines and different types of tests, all of them were consistent with

lower toxicity of individual OTA derivatives. Our studies also indicated lower embryotoxicity in chicks.

In conclusion, the presented study demonstrated that according to the in ovo tests on chicken embryos, the racemic form of OTA contributed to embryonic mortality to a lower extent than OTA injected eggs before incubation at the same dose. The liver, kidneys and yolk sac were the main body parts that accumulated the toxins, with higher concentrations of OTA than 2'R-OTA detected in such organs as liver and kidneys. However, a higher concentration of 2'R-OTA than that of OTA was found in blood serum and hearts. The obtained results showed that there were differences in the ability to accumulate toxins in certain organs, which, to a certain extent, affected the potential toxicity on individual organs. Also, the cytotoxicity assessment of OTA and its derivatives by means of MTT assay toward chicken embryonic cell lines suggested higher OTA toxicity than the remaining compounds. In order to inhibit cell metabolic activity to a comparable degree, 2'R-OTA concentration must be 27 times higher than that of OTA. A similar observation was reported for the remaining compounds (OTB and OTα).

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DISCLOSURES

The authors declare no conflict of interest. None of the authors of this study has a conflict of interest related to employment, consultancies, grants, or other funding.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102413.

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