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The individual and combined effects of ochratoxin A with citrinin and their metabolites (ochratoxin B, ochratoxin C, and dihydrocitrinone) on 2D/3D cell cultures, and zebrafish embryo models

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ABSTRACT

Ochratoxin A and citrinin are nephrotoxic mycotoxins produced by *Aspergillus*, *Penicillium*, and/or *Monascus* species. The combined effects of ochratoxin A and citrinin have been examined in more studies; however, only limited data are available regarding the co-exposure to their metabolites. In this investigation, the individual toxic effects of ochratoxin A, ochratoxin B, ochratoxin C, citrinin, and dihydrocitrinone were tested as well as the combinations of ochratoxin A with the latter mycotoxins were examined on 2D and 3D cell cultures, and on zebrafish embryos. Our results demonstrate that even subtoxic concentrations of certain mycotoxins can increase the toxic impact of ochratoxin A. In addition, typically additive effects or synergism were observed as the combined effects of mycotoxins tested. These observations highlight that different cell lines (e.g. MDBK vs. MDCK), cell cultures (e.g. 2D vs. 3D), and models (e.g. *in vitro* vs. *in vivo*) can show different (sometimes opposite) impacts. Mycotoxin combinations considerably increased miR-731 levels in zebrafish embryos, which is an early marker of the toxicity on kidney development. These results underline that the co-exposure to mycotoxins (and/or mycotoxin metabolites) should be seriously considered, since even the barely toxic mycotoxins (or metabolites) in combinations can cause significant toxicity.

1. Introduction

Mycotoxins are common toxic contaminants in food, drinks, and animal feed. Ochratoxins are produced by *Aspergillus* and *Penicillium* molds, they typically occur in cereals, meat products, fruits, spices, and beverages (e.g., wine, beer, and coffee) (Di Stefano et al., 2015; Kőszegi and Poór, 2016; Malir et al., 2016). Ochratoxins are built up from dihydroisocoumarin and L-phenylalanine structures (Fig. 1). The most abundant food contaminant is ochratoxin A (OTA); while its metabolites, ochratoxin B (OTB; the dechlorinated derivative of OTA) and ochratoxin C (OTC; the ethyl ester analogue of OTA), are less frequent in the food chain (Heussner and Bingle, 2015; Kőszegi and Poór, 2016; Kumar et al., 2020). OTA is a nephrotoxic mycotoxin, but its hepatotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic, and carcinogenic effects have also been reported (Gupta et al., 2018; Kőszegi and Poór, 2016; Malir et al., 2016). The International Agency for Research on Cancer (IARC) classifies OTA as a possible human carcinogen (group 2B) (IARC, 1993). Previous investigations suggested the lower toxicity of OTB vs. OTA (Heussner and Bingle, 2015; Kumar et al., 2020). However, the results are controversial regarding OTC: earlier studies demonstrated its lower, similar, or even higher toxic effects compared to OTA (Köhler et al., 2002; Müller et al., 2003a, 2003b; Xiao et al., 1996).

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Citrinin (CIT; Fig. 1) is also a nephrotoxic mycotoxin produced by *Aspergillus*, *Penicillium*, and *Monascus* species (EFSA, 2012). CIT frequently occurs in cereals, fruits, vegetables, nuts, rice, and the corresponding products. In addition, red mold rice (food colorant, flavor enhancer, and meat preservative applied in some Asian countries) can also be a relevant source of CIT exposure (EFSA, 2012). Similar to OTA, CIT may contribute to the development of endemic nephropathy in humans and pigs (Flajs and Peraica, 2009; Peraica et al., 2008). Dihydrocitrinone (DHC; Fig. 1) is the major urinary metabolite of CIT (Ali et al., 2018; Gerding et al., 2015), it is less toxic compared to the parent mycotoxin (Faisal et al., 2018; Föllmann et al., 2014).

Penicillium and *Aspergillus* fungi can produce both ochratoxins and CIT. Therefore, the co-exposure to these mycotoxins is common (Gupta et al., 2018). Previous studies also highlight the simultaneous presence of OTA and CIT in food and animal feed samples (Ali, 2018; Molinié et al., 2005; Pleadin et al., 2018; Vrabcheva et al., 2000). Furthermore, the co-occurrence of OTB and OTC with OTA has been reported in wine samples (Di Stefano et al., 2015; Remiro et al., 2012), which can be explained by the metabolism of OTA by *Saccharomyces cerevisiae* (Freire et al., 2019). In addition, the simultaneous presence of OTA, CIT, and DHC was also described in human blood samples (Ali et al., 2018; Blaszkewicz et al., 2013). Therefore, it is reasonable to consider the possible risk of the co-exposure to OTA, CIT, and/or their metabolites.

The combined effects of two compounds can be antagonism, additive impact, or synergism. If the combined action is lower than the sum of the individual effects, then it is called as antagonism. Additive impact can be observed if the combined action is similar than the sum of the individual effects. Furthermore, synergism is evoked when the combined impact is significantly larger compared to the sum of the individual impacts. Both *in vitro* and *in vivo* studies revealed that the co-exposure to OTA and CIT typically results in additive effect or synergism, while certain studies suggested antagonism (Föllmann et al., 2014; Heussner and Bingle, 2015; Klarić et al., 2013; Speijers and Speijers, 2004). The few data are controversial regarding the combined effects of OTA and OTB (Heussner et al., 2006; Lea et al., 1989; Roth et al., 1989; Størmer et al., 1985). In a previous study, no synergism was observed between OTA and OTC in THP-1 (human monocyte/macrophage) cells (Müller et al., 2003b). Furthermore, the co-exposure to DHC with other mycotoxins has not been examined yet.

Zebrafish embryo is an ideal vertebrate model animal for studying developmental toxicology (Braunbeck et al., 2005; Scholz et al., 2008) because the developing and transparent zebrafish can be assessed conveniently for lethality and developmental abnormalities from fertilization through larval stages (Weigt et al., 2011). In addition, the patterning of the pronephric nephrons in zebrafish is similar to the metanephric nephrons of mammals (Desgrange and Cereghini, 2015; Wingert and Davidson, 2008). Therefore, zebrafish models are particularly suitable for the investigation of nephrotoxic substances (Wu et al., 2016). MicroRNAs (miRNAs) are a class of small noncoding RNA molecules, they are built up from ~22 nucleotides and negatively regulate gene expression. The exposure to toxins and xenobiotics can change miRNA expression profiles resulting in various physiological alterations (Tai and Freeman, 2020). Therefore, miRNAs became popular biomarkers in the prediction of xenobiotic-related risk. In zebrafish, miR-731 is a suitable biomarker to predict and assess the nephrotoxic effects of OTA (Wu et al., 2016).

Considering the frequent co-occurrence of ochratoxins and CIT, the individual impacts of OTA, OTB, OTC, CIT, and DHC (Fig. 1) were investigated on MDBK (kidney, bovine) and MDCK (kidney, canine) cell lines, employing two-dimensional (2D) and three-dimensional (3D) models. Thereafter, the combined toxic effects of OTA with the other mycotoxins listed were examined and evaluated. Moreover, the individual and combined toxicity of mycotoxins was also tested in zebrafish embryo models.

2. Materials and methods

2.1. Reagents

Ochratoxin A (OTA), citrinin (CIT), MEM (Minimum Essential Medium with Earle's Balanced Salts, with 2.0 mM L-glutamine, with non-essential amino acids, without sodium bicarbonate), and DMEM (Dulbecco's modified Eagle medium) were purchased from Merck (Darmstadt, Germany). Ochratoxin B (OTB), ochratoxin C (OTC), and dihydrocitrinone (DHC) were obtained from Cfm Oskar Tropitzsch (Marktredwitz, Germany), Cayman Chemical Company (Ann Arbor, MI, US), and AnalytiCon Discovery (Potsdam, Germany), respectively. L-glutamine, Hepes buffer, and non-essential amino acids were from Lonza (Basel, Switzerland). Fetal bovine serum (FBS; Pan-Biotech, Aidenbach, Germany), β-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, US), bioluminescent ATP Assay Kit CLSII (Roche, Basel, Switzerland), and CellTiter Glo 3D kit (Promega, Charbonnières-les-Bains, France) were used as received. Mycotoxin stock solutions (each 5000 μM) were prepared in 96 v/v% ethanol (spectroscopic grade; VWR, Debrecen, Hungary) and stored at −20 °C.

2.2. 2D cell models employing MDBK and MDCK cell lines

MDBK (bovine kidney, epithelial, adherent, ATCC: CCL-22) cells were cultured in MEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). MDCK (canine, kidney, epithelial, adherent, ATCC: CCL-34) cells were cultured in DMEM (with high glucose, 4500 mg/L) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Both cell lines were cultured in a

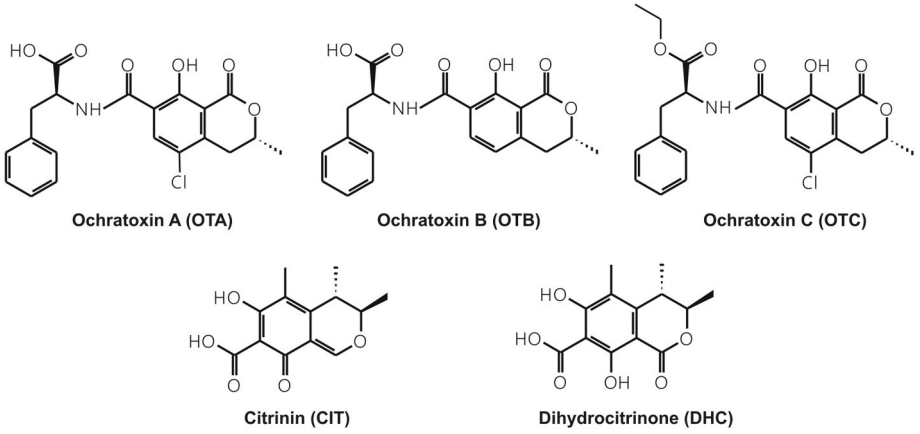

Fig. 1. Chemical structures of ochratoxin A (OTA), ochratoxin B (OTB), ochratoxin C (OTC), citrinin (CIT), and dihydrocitrinone (DHC).

humidified atmosphere (5% CO_2), in 75 cm^2 sterile cell culture flasks at 37 °C. Trypsinized cell suspensions were plated into 96-well sterile plastic plates. After 24 h (directly before the treatment), cell culture medium was replaced with fresh one without FBS. Ochratoxins (Faisal et al., 2020; Kőszegi and Poór, 2016), CIT (Poór et al., 2015), and DHC (Faisal et al., 2019) form highly stable complexes with serum albumin. Therefore, to exclude the effects of albumin-ligand interactions (e.g., competition or displacement), we performed the treatments in the absence of FBS. To examine the individual toxic impacts of mycotoxins (0–50 μM), cells were treated for 24 h. To test the combined effects of OTA with OTB, OTC, CIT, and DHC, subtoxic and/or toxic concentrations of the latter mycotoxins were added to standard amount of OTA (which caused approximately 20% decrease in ATP levels). Solvent controls were applied in each experiment, where 1.0 v/v% ethanol was the highest concentration used.

The changes in cell viability were evaluated based on intracellular ATP levels. Since ATP content is an indicator of metabolically active cells, ATP concentration is a suitable parameter to assess the number of viable cells (Adan et al., 2016; Kocyigit et al., 2018; Lundberg et al., 2020). In addition, our previous studies also demonstrated that ATP is a suitable parameter to evaluate the ochratoxin-, CIT-, and DHC-induced loss in cell viability (Csepregi et al., 2018; Faisal et al., 2019, 2020; Kőszegi and Poór, 2016; Poór et al., 2015). ATP concentrations were determined with the luciferin-luciferase reaction as it has been reported (Csepregi et al., 2018), without modifications. The corresponding figures in the manuscript represent ATP concentrations/well (%) compared to the control.

To confirm the results of ATP measurements, total protein concentrations were also determined in 2D cell experiments, applying fluorescamine for the fluorescent derivatization of proteins (Csepregi et al., 2018). Cells were lysed (200 μL/well) in borate buffer (0.2 M, pH 9.2) containing 0.1% Triton X-100 and 20 mM ethylenediaminetetraacetic acid. Lysed samples (20 μL/well) were pipetted into a new 96-well plate, then 150 μL/well lysis buffer and 50 μL/well fluorescamine solution (0.3 mg/mL dissolved in acetone) were added. After the short shaking of samples, fluorescence signals (λ_{ex} = 385 nm; λ_{ex} = 490 nm) were measured using an Enspire Multimode plate reader (PerkinElmer, Waltham, MA, US). Total protein data were calculated applying a bovine serum albumin (Biosera, Nuaille, France) calibration curve. The corresponding figures in the supplementary (Figs. S1 and S2) demonstrate total protein concentrations/well (%) compared to the control.

2.3. 3D cell model (MDCK cell line)

MDCK cells were cultured in DMEM (supplemented with 10% FBS, penicillin, streptomycin, L-glutamine, HEPES buffer, non-essential amino acids, and β-mercaptoethanol). MDCK cells (10^6) were seeded in 75 cm^2 flasks and incubated at 37 °C in a humidified atmosphere (5% CO_2). The medium was changed every 2 days. After 3–4 days (80–90% confluency), cells were trypsinized. For spheroid generation, a cell suspension (100 μL/well) in DMEM was used containing 1% FBS at optimized density of 10^4 cells/well. Cells were dispensed into 96-well ultra-low attachment plates (Corning, Boulogne-Billancourt, France). After 3 days, 100 μL FBS-free medium was added to each well, then spheroids were treated at day 5 with the mycotoxins. Images were captured using EVOS FL Cell Imaging System (Thermo Fisher Scientific). After 24 h treatment, cell viability was determined using a CellTiter Glo 3D kit (Promega), according to the manufacturer's instructions. ATP luminescence was measured using an Enspire Multimode Plate Reader (PerkinElmer, Waltham, MA, US). The preparation of spheroids and the treatment of the 3D cell culture are summarized in Fig. S3. Solvent controls were applied in each experiment (1.0 v/v% ethanol was the highest concentration used).

2.4. Experiments on zebrafish embryos

Laboratory-bred AB strain and Tg(wt1b:GFP) (Fritz Lipmann Institute, Gemany) transgenic zebrafish (Perner et al., 2007) were held in breeding groups of 30 females and 30 males at the Department of Ecotoxicology (Hungarian University of Agriculture and Life Sciences, Hungary), in a Tecniplast ZebTEC recirculation system (Tecniplast S.p.A., Buguggiate, Italy) at 25.5 ± 0.5 °C, pH 7.0 ± 0.2, conductivity 550 ± 50 mS (system water) and light:dark period of 14 h:10 h. Fish were fed twice a day with dry granulate food (Zebrafeed 400–600 mm; Sparos Lda., Olhão, Portugal) supplemented with freshly hatched live *Artemia salina* twice a week. Fish were placed in breeding tanks (Tecniplast S.p.a.) late in the afternoon the day before the experiment, and allowed to spawn by removing the dividing walls next morning. The collected eggs were incubated in system water (26 ± 1 °C) in Petri dishes until the start of the experiments.

The tests were performed on newly fertilized AB zebrafish eggs and the exposure started before the embryos reached eight-cell stage. The following test concentrations were used: OTA (1.0, 0.5, 0.4, 0.3, 0.25, 0.2, 0.1, 0.05, and 0.025 μM), OTB (25, 12.5, 6.25, 5.5, 5.0, 4.0, 3.125, 1.56, and 0.78 μM), OTC (3.125, 1.56, 0.78, 0.39, 0.19, and 0.09 nM), CIT and DHC (50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 μM). Embryos in groups of five in four replicates (20 embryos) were treated with the listed concentrations of mycotoxins, using static conditions. All tests were carried out in 24-well microtiter plates (2 mL solution/well). Embryos were treated at 26 ± 1 °C with a 14 h:10 h light:dark period. Mortality values for embryos were determined at 120 hpf (hours past fertilization) on the basis of egg coagulation, based on the lack of somite formation and the lack of heart function. LC_{50}, LC_{25}, LC_{10}, $LC_{2.5}$, and LC_1 values were determined from the mortality data.

In the co-exposure experiments, OTA was applied at 0.23 μM fixed concentration (LC_{25}). Concentrations of OTB (3.78, 3.21, 2.58, and 2.25 μM) and OTC (0.25, 0.19, 0.14, and 0.11 nM) were selected based on the LC_{25}, LC_{10}, $LC_{2.5}$, and LC_1 values determined previously. Since CIT and DHC did not cause mortality even at 50 μM concentration, their three sublethal concentrations (25, 12.5, and 3.125 μM) were applied. The experimental setup was the same as described for the acute embryo toxicity experiment. Mortality values and sublethal effect of the treatments were determined after 120 h exposure.

OTA (0.23 μM), OTB (2.25 μM), OTC (0.055 nM), CIT (3.125 μM), and DHC (3.125 μM) were examined individually as well as the same concentrations of OTB, OTC, CIT, and DHC were tested in combinations with OTA (0.23 μM) on transgenic Tg(wt1b:GFP) zebrafish embryos. Treatments were started before the embryos reached eight-cell stage, embryos were kept under static conditions for 48 h. Embryos in groups of twenty in three replicates (60 embryos) were tested in 6-well plates. Each well was filled with 10 mL solution and larvae were incubated at 26 ± 1 °C with 14 h:10 h light:dark cycle. Mortality values and kidney morphology were determined at the end of the exposure.

Total RNA from Tg(wt1b:GFP) embryos was isolated using Tri-Reagent (Merck) and chloroform (Carlo Erba, Barcelona, Spain). RNA was precipitated in isopropanol (Fisher Scientific, Hampton, NH, US) and then the pellet was washed with 75% ethanol (Fisher Scientific) and dissolved in RNase-free water (Ambion, Austin, TX, US). Concentration and quality were measured by NanoDrop (Thermo Fisher Scientific). To eliminate DNA contamination, RNA samples were treated with DNase (Thermo Fisher Scientific). For miR-731 (MIMAT0003761) quantification, cDNA was produced with specific stem-loop primers designed by Wu et al. (2016), based on the method of Varkonyi-Gasic et al. (2007). The miR-26a was used as a reference gene. Quantitative PCR reactions were assembled in 20 μL final volume containing miRNA (miR-731 or miR-26a) specific forward primer and universal miRNA reverse primer (each 250 pM) (primer sequences are demonstrated in Tables S1) and 1x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne, Tartu, Estonia) and 2 μL diluted cDNA as a template or PCR-grade water as a negative control. PCR reactions were run in a StepOnePlus real-time PCR

system (Applied Biosystems, Waltham, MA, US) with the following protocol: 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 30 s. Dissociation protocols were used to measure melting curves. The relative abundance of target genes was calculated using the following formula: $R = 2^{-\Delta\Delta Ct}$. Data were statistically evaluated using GraphPad Prism8 software (GraphPad Software Inc., San Diego, CA, US).

Prior to microscopic analysis, embryos were anaesthetized in 0.02% MS-222 (tricaine methanesulphonate; Merck) solution, then larvae were oriented in 0.5% methyl-cellulose. Bright field images from wild type embryos were captured in 30 × magnification, fluorescent images were captured from transgenic embryos in 50 × magnification (Leica M205 FA, Leica DFC 425C camera, LAS V3.8 software). Regarding transgenic embryos (48 hpf), the eggshell was gently removed using a fine forceps, this was not necessary for wild-type embryos (120 hpf).

All studies were completed before the treated individuals reached free-feeding stage. Experiments were performed in accordance with the Hungarian Animal Welfare Law (XIV-I-001/2303–4/2012) and the European directive (2010/63/EU) on the protection of animals used for scientific purposes.

2.5. Evaluation of combined effects

To evaluate the combined effects of OTA with OTB, OTC, CIT, and DHC, the combination index (*CI*) values were determined employing the CompuSyn software (ComboSyn Inc., Paramus, NJ, US) based on the median-effect equation of the mass-action law (Chou and Talalay, 1984):

$$log\frac{f_a}{f_u} = m \times \log(D) - m \times log(D_m) \quad (1)$$

where f_a and f_u denote the affected and unaffected fraction of the system, respectively; D is the dose, D_m represents the dose necessary to evoke the median effect, while m stands for the sigmoidicity coefficient ($m > 1$ sigmoidal, $m = 1$ hyperbolic, $m < 1$ flat sigmoidal).

The CI values were determined based on the following equation (Chou and Talalay, 1984):

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad (2)$$

where $(D_x)_1$ and $(D_x)_2$ are the doses of compound 1 and compound 2 alone to exert x% effect, while $(D)_1$ and $(D)_2$ are the doses of the two compounds in combination to evoked the same effect as $(D_x)_1$ alone or $(D_x)_2$ alone, i.e. the targeted x% effect. Additive effect can be considered if the CI value is approximately 1 ($0.8 < CI < 1.2$), while $CI < 0.8$ or $CI > 1.2$ denote synergism or antagonistic effect, respectively (Balázs et al., 2021).

2.6. Calculation of IC_{50}/LC_{50} values and statistical analyses

IC_{50}/LC_{50} values were determined by sigmoidal fitting employing the GraphPad Prism 8 software. Figures represent the mean as well as the standard error of the mean (±SEM) values at least from three independent experiments. To establish the statistical significance ($p < 0.05$ and $p < 0.01$), one-way ANOVA (with Tukey post hoc) test was performed, applying SPSS Statistics 26.0 (IBM, Armonk, NY, US).

3. Results and discussion

3.1. Individual in vitro toxic effects of mycotoxins

Since both OTA and CIT are nephrotoxic mycotoxins (Gupta et al., 2018), the individual toxic effects of OTA, OTB, OTC, CIT, and DHC were investigated on kidney cell lines (MDBK and MDCK), employing 2D and 3D models. Ochratoxins form extremely stable complexes with serum albumin (K_a values are typically 10^6–10^7 L/mol) (Faisal et al., 2020; Kőszegi and Poór, 2016). Furthermore, CIT (Poór et al., 2015) and DHC (Faisal et al., 2019) also bind to the protein with high affinity (K_a values are approximately 10^5 L/mol). We aimed to examine the toxicodynamic interactions of mycotoxins tested; therefore, to exclude the involvement of albumin-ligand interactions, our experiments were performed without FBS (or with 0.5% FBS in 3D experiments, where the presence of FBS was indispensable for the appropriate spheroid generation). In each model used, OTA induced strong concentration-dependent decrease in ATP levels, showing 0.9 μM (MDBK, 2D), 1.6 μM (MDCK, 2D), and 1.7 μM (MDCK, 3D) IC_{50} values (Fig. 2). Regarding OTB, MDBK cell line was more sensitive compared to the MDCK cells, and the 3D cell culture was less vulnerable vs. the 2D cell culture. In each model, OTB represented much lower toxicity than OTA. Among mycotoxins tested, OTC caused the highest toxicity in both cell lines used, showing lower toxicity in MDBK than in MDCK cell line (Fig. 2). Interestingly, the toxic impacts of OTA and OTC were similar in MDBK cells, while the latter mycotoxin showed considerably higher toxicity in MDCK (2D and 3D) models. CIT demonstrated lower toxic effect than ochratoxins (except the MDCK 3D model, where it showed similar impact to OTB). CIT induced strong decrease in ATP levels only in MDBK cells (at 25 and 50 μM concentrations); however, it was less toxic in MDCK cell line (Fig. 2). DHC did not affect ATP concentrations in the models applied. OTA, OTB, and CIT showed higher toxicity in the 2D vs. the 3D MDCK model, while we noticed the opposite effect for OTC (Fig. 2).

In 2D cell experiments, total protein levels were also analyzed. Mycotoxins induced similar or somewhat lower relative decreases in total protein levels (Fig. S1) compared to ATP concentrations (Fig. 2). OTA (and likely its metabolites) can decrease cellular ATP levels, the mechanism is complex and not fully understood yet (Khoi et al., 2021; Kőszegi and Poór, 2016). ATP depletion may explain the larger reduction of ATP (vs. total protein) levels at certain concentrations of mycotoxins, and confirms that ATP is a sensitive indicator of ochratoxin-mediated toxicity.

The acute *in vitro* toxicity of OTA has been extensively studied, however, less data is available regarding OTB and OTC. In agreement with our results, previous *in vitro* studies are consistent that OTB exerts significantly lower toxic effect than OTA (Bondy and Armstrong, 1998; Bruinink et al., 1997; Dietrich et al., 2001; Xiao et al., 1996). Furthermore, OTB showed higher toxicity than CIT on LLC-PK1 and opossum kidney cells (Bondy and Armstrong, 1998), and it was less toxic compared to OTC on HepG2 cell line (Faisal et al., 2020). OTA showed higher toxic effect than OTC on HeLa cells (Xiao et al., 1996), while OTC was more toxic compared to OTA on *Bacillus brevis* (Xiao et al., 1996) as well as on kidney cell lines (Müller et al., 2003b), monocytes (Müller et al., 2003a), and on swine peripheral blood mononuclear cells (Köhler et al., 2002). The latter observations are in agreement with our results (Fig. 2).

The lower toxicity of CIT vs. OTA has been demonstrated on several cell lines (Bouslimi et al., 2008a, 2008b; Föllmann et al., 2000; Gong et al., 2019; Knecht et al., 2005). Furthermore, earlier *in vitro* studies suggested that DHC is a less toxic metabolite of CIT (Faisal et al., 2018; Föllmann et al., 2014).

3.2. Combined in vitro toxic effects of OTA with OTB, OTC, CIT, and DHC

The combined toxic effects of OTA with subtoxic and/or toxic concentrations of OTB, OTC, CIT, and DHC were also investigated. In these studies, OTA was applied at one fixed concentration (that induced approximately 20% decrease in ATP levels).

In MDBK (2D) cell line, the higher applied subtoxic concentrations of OTC, CIT, and DHC significantly increased the OTA-induced toxicity, while OTB did not affect it (Fig. 3A). However, in MDCK (2D) cells, only DHC enhanced the toxic action of OTA (Fig. 3B). Interestingly, the

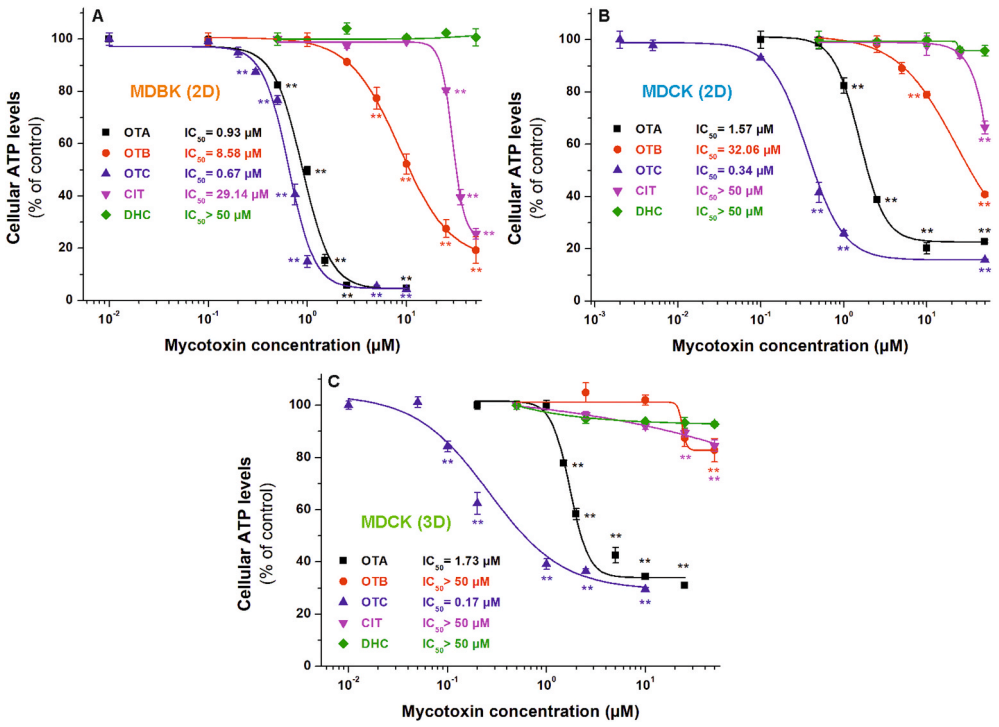

Fig. 2. Effects of OTA, OTB, OTC, CIT, and DHC on MDBK 2D (**A**), MDCK 2D (**B**), and MDCK 3D (**C**) cells (ATP/well, % of control; **p < 0.01).

MDCK 3D model showed remarkable differences compared to the 2D models: The lower concentrations of OTB (2.5 and 5 μM) decreased, while its higher subtoxic concentration (25 μM) increased the OTA-mediated toxicity (Fig. 3C). The lower subtoxic concentration of OTC (0.01 μM) also reduced the toxic effect of OTA, while the co-treatment with 0.05 μM OTC did not change the OTA-induced impact. The co-exposure to OTA with subtoxic concentrations of CIT (2.5 and 5 μM) and DHC (2.5, 5, 25, and 50 μM) resulted in major decreases in ATP levels (Fig. 3C). These observations suggested that the 3D cell culture may be a complex and suitable model for the more precise evaluation of mycotoxin co-exposures. As it has been demonstrated in earlier studies, when cells are cultured in 3D spheroids, the cellular physiology is significantly altered compared to the standard 2D cultures (Fennema et al., 2013; Foglietta et al., 2020; Jensen and Teng, 2020; Lee et al., 2020). This modification of cell cultures provides physiological processes which are much closer to the *in vivo* systems, because the 3D spheroid formation reestablishes intercellular connections and matrix components as well as restores/improves drug transporter and metabolic enzyme activities. Taken together, 3D spheroid cultures are powerful tools to get more reliable data from *in vitro* experiments in pharmacological and toxicological investigations. Importantly, DHC showed negligible effects in the single compound tests, while it strongly increased the OTA-induced toxicity in each *in vitro* model (Fig. 3A–C). In our previous study, the barley toxic 2′R-ochratoxin A also significantly increased the OTA-induced toxicity in MDCK cells (Faisal et al., 2018).

Employing the CompuSyn software (see in 2.5), the CI values were determined and the combined effects of mycotoxins have been evaluated. When cells were co-treated with OTA and toxic concentrations of OTB, OTC, and CIT, typically additive effect and/or synergism were observed (Fig. 3D–F). OTB showed antagonism or additive impact in 2D cell cultures, while synergism was noticed in the 3D model. OTC induced additive action or synergism in MDBK cell line; however, its synergistic effects were noticed in MDCK (both 2D and 3D) models. CIT showed additive impact in MDBK 2D and MDCK 3D models, while it caused synergism in MDCK 2D model.

In 2D models, total protein levels were also determined. Interestingly, the relative changes in ATP and protein concentrations were very similar in MDCK cells, while we noticed lower mycotoxin-induced decreases in the protein content (vs. the ATP) in MDBK cells (see in Fig. S2 and Fig. 3). Nevertheless, the relative changes of ATP and total protein levels showed similar tendencies.

In previous *in vitro* studies, antagonism (Lea et al., 1989), additive effect (Roth et al., 1989), and synergism (Heussner et al., 2006) have also been reported as a result of the co-treatment with OTA and OTB. Furthermore, OTB inhibited the OTA-induced toxic impacts in rats (Størmer et al., 1985). The combined effects of OTA and OTC are barely examined; nevertheless, no synergism was observed on THP-1 cells (Müller et al., 2003b).

The combined effects of OTA and CIT have been examined in several studies, showing controversial results (Klarić et al., 2013; Speijers and Speijers, 2004; Schulz et al., 2018). The co-treatment with OTA and CIT induced synergism on pig lymphocytes (Bernhoft et al., 2004) as well as on Vero (Bouslimi et al., 2008a, 2008b, 2008b), LLC-PK1 (Heussner et al., 2006), HepG2 (Gayathri et al., 2015), and HEK293 cell lines (Gong et al., 2019). Furthermore, in human proximal tubule (HK-2) cells, nanomolar OTA and CIT acted synergistically in nephropathic processes (Schulz et al., 2018). In contrast, low concentrations of CIT did not affect the OTA-induced impact, few micromolar levels of CIT (2.5 and 5.0 μM) antagonized the apoptotic effect of OTA (25 and 50 nM), while the co-exposure to higher CIT concentrations (7.5 and 15 μM) provoked additive effects on immortalized human proximal tubule cells (Knecht et al., 2005). In another study, no combined effects of OTA and CIT were noticed on V79 cells (Föllmann et al., 2000). Based on our current knowledge, the co-treatment of DHC with other mycotoxins has not been tested previously.

3.3. *Individual effects of mycotoxins on zebrafish embryos – Mortality data*

Among the five substances tested, OTC was highly the most toxic (LC_{50} = 0.32 nM; 120 h exposure), followed by OTA (LC_{50} = 0.28 μM) and OTB (LC_{50} = 4.46 μM). CIT and DHC did not cause mortality even at 50 μM concentrations (Fig. 4).

Previous *in vivo* studies also suggested the lower toxicity of OTB vs.

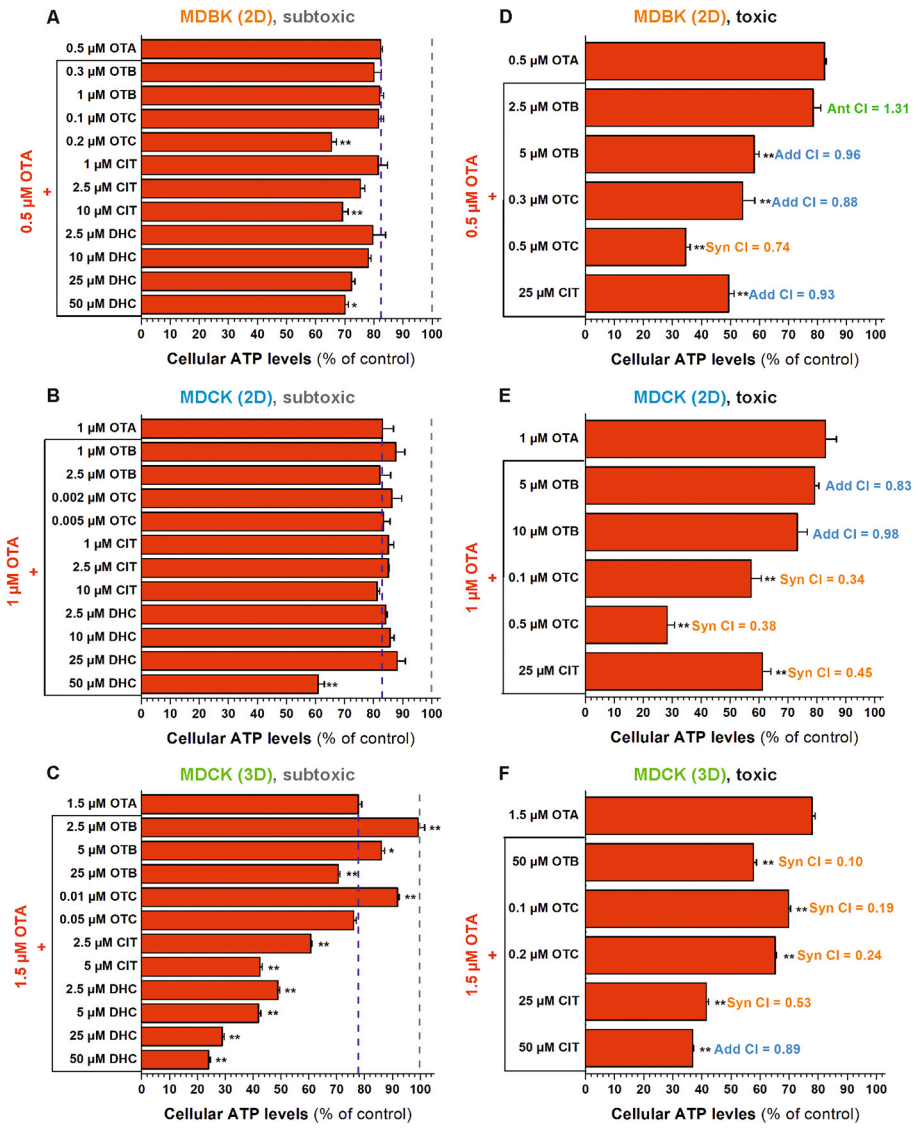

Fig. 3. Effects of OTA alone and in combination with the subtoxic and/or toxic concentrations of OTB, OTC, CIT, and DHC on MDBK 2D (A and D, respectively), MDCK 2D (B and E, respectively), and MDCK 3D (C and F, respectively) models (ATP/well, % of control; compared to the effect OTA alone: *p < 0.05, **p < 0.01).

OTA, which is partly resulted from its faster elimination (Heussner and Bingle, 2015; Li et al., 1997; Mally et al., 2005). Furthermore, the higher toxicity of OTA compared to CIT has also been reported (Gupta et al., 2018; Kumar et al., 2007).

3.4. *Effects of mycotoxin combinations on zebrafish embryos – Mortality data*

Combined toxicity of mycotoxins was also tested on zebrafish embryos. LC_{25} of OTA (0.23 µM) was applied as its fixed concentration. LC_1 and $LC_{2.5}$ values were used as subtoxic levels of OTB and OTC, while LC_{10} and LC_{25} as their toxic concentrations (Table S2). Due to their low toxicity in zebrafish embryos, CIT and DHC were used only at subtoxic concentrations (3.125, 12.5, and 25 µM).

Generally, mycotoxin pairs caused higher mortality than OTA alone (Fig. 5). Even the lowest subtoxic concentrations of OTB (2.25 µM) and OTC (0.11 nM) applied increased the mortality, resulting in low or even zero survival (Fig. 5A). Furthermore, significant increases in mortality were also observed in the presence of CIT (25 µM) or DHC (12.5 and 25 µM).

When the toxic concentrations of OTB were combined with OTA, additive effect or synergism were noticed, while OTC caused synergism at both toxic concentrations tested (Fig. 5B). The combination of OTA (0.23 µM) with higher OTB (3.78 µM) or both OTC (0.19 and 0.25 nM) levels induced 100% mortality.

3.5. *Effects of mycotoxins and mycotoxin combinations on zebrafish embryos – Sublethal symptoms*

Sublethal symptoms of mycotoxins were also examined at the end of the exposure period. Phenotypic malformations were much more pronounced in co-exposure studies compared to individual mycotoxin treatments (Fig. 6). Individual treatments and co-exposures to mycotoxins inhibited swim bladder opening in zebrafish embryos. OTA caused the typical malformations described in the literature, including abnormal yolk shape and color, slight dorsal curve of the body axis, and pericardial edema (Csenki et al., 2019; Haq et al., 2016; Wu et al., 2016). The individual treatment with OTB or OTC induced changes in the shape and color of the yolk and pericardial edema. In addition, OTB caused the distortion of the head region. The exposure to 50 µM CIT led to pericardial edema, blood accumulation, incorrect heart looping, and reduced the size of cardiac chambers, which is in agreement with the previously reported observations (Wu et al., 2013). Nevertheless, the 25 µM concentrations of CIT and DHC (individual treatments) did not

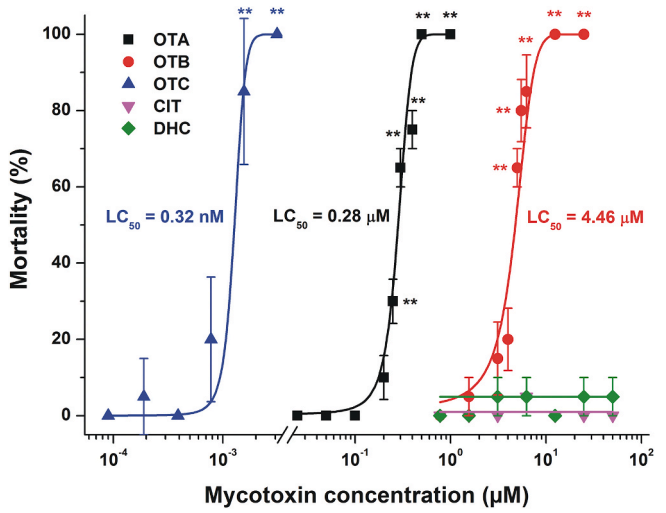

Fig. 4. Concentration-response curves of individually tested mycotoxins for mortality in 120 hpf zebrafish embryos. The order of toxicity after 120 h exposure: OTC > OTA > OTB > CIT ≈ DHC.

induce phenotypic differences compared to the control group, except the lack of swim bladder opening (Fig. 6). Besides the above-listed symptoms, the co-exposure to OTA with OTB resulted in the backward curvature of the body axis. The combined treatment with OTA and OTC caused remarkable, whole-body malformations (Fig. 6). When zebrafish embryos were treated simultaneously with OTA-CIT or OTA-DHC, the changes in yolk shape, a gray discoloration as well as pericardial and yolk edemas were also appeared. In addition, the co-exposure to OTA with CIT induced the curved body of the embryos in the dorsal direction, while DHC resulted in a curvature slightly towards the abdominal direction (Fig. 6).

3.6. *Effects of mycotoxins and mycotoxin combinations on the expression of miR-731 and the pronephros of zebrafish embryos*

In zebrafish, OTA partly exerts its adverse effects on kidney development through the enhancement of miR-731 expression; therefore, miR-731 is a suitable biomarker of OTA-induced nephrotoxic effects (Wu et al., 2016). The individual and combined *in vivo* nephrotoxic effects of mycotoxins were examined based on their impacts on the expression of miR-731 and the morphological changes of the pronephros, using low mycotoxin concentrations. In a previous study, the treatment of zebrafish embryos with 0.5 µM OTA led to a very high increase in expression after 48 h exposure, while 0.25 µM OTA did not change the expression of miR-731 significantly (Wu et al., 2016). Therefore, we maintained a baseline concentration of OTA (0.23 µM), which did not cause significant increase in miR-731 expression compared to the control (Fig. 7A). Regarding the other mycotoxins, one sublethal concentration was selected (OTB: 2.25 µM; OTC: 0.055 nM; CIT: 3.125 µM; DHC: 3.125 µM) based on the individual toxin assays (Fig. 4). After 48 h exposure, the individual treatments with mycotoxins did not affect significantly the expression of miR-731 in the embryos, while the combination of OTA with the other mycotoxins strongly increased miR-731 levels (Fig. 7A). The largest increase was induced by the OTA-OTC mixture, followed by OTA-OTB, OTA-CIT, and OTA-DHC.

Since miR-731 expression clearly influences kidney development in zebrafish embryos (Wu et al., 2016), the phenotypical consequences were also examined. We used a transgenic zebrafish line Tg(wt1b:GFP) expressing green fluorescent protein in the pronephros (Perner et al., 2007). In agreement with the miR-731 expression data (Fig. 7A), there is no significant difference in pronephros morphology between individual toxin treatments and the control (Fig. 7B). However, the combined treatment of zebrafish embryos with OTA and other mycotoxins tested led to visible phenotypic changes on the overall structure of the pronephros. The most significant mycotoxin-induced malformations were observed in the pronephric duct section of the pronephros, while the podocytes of renal corpuscle and tubules remained relatively intact (Fig. 7B). These observations confirm that the co-exposure to these nephrotoxic mycotoxins may have significant effects on renal function and development even at relatively low concentrations.

We did not find data regarding the plasma concentrations of OTB and OTC in humans. Previous reports suggested low levels of OTA, CIT, and DHC in the human circulation: OTA can reach 100 nM or even higher plasma concentrations in endemic regions (Kőszegi and Poór, 2016), while CIT and DHC levels are typically at the low nanomolar range (Blaszkewicz et al., 2013; Ali et al., 2018; Faisal et al., 2019). In our cell and zebrafish experiments, the higher concentrations of these mycotoxins were applied, because we tested their acute toxic effects. OTA, CIT, and their metabolites may cause human toxicity as a result of the chronic exposure; however, the *in vitro* modeling of these long-term effects is very difficult. Furthermore, OTA (Kőszegi and Poór, 2016) and likely CIT (Berndt and Hayes, 1982) are actively taken up by organic anion transporters, which may result in the gradual accumulation of these mycotoxins and their metabolites in kidney tubules. Therefore, this study provides important data, even if the mycotoxin concentrations used were considerably higher than the typical human plasma concentrations described in the scientific literature.

4. Conclusions

In the current study, the individual and combined effects of OTA, OTB, OTC, CIT, and DHC were tested. For the complex evaluation, two kidney cell lines (MDBK and MDCK), 2D and 3D cell cultures, and

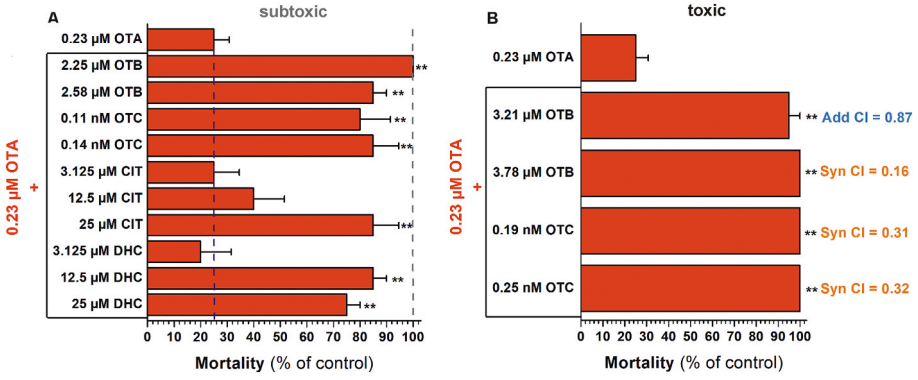

Fig. 5. Mortality caused by OTA (0.23 µM) alone and in combination with the subtoxic (A) and/or toxic (B) concentrations of OTB, OTC, CIT, and DHC on 120 hpf zebrafish embryos.

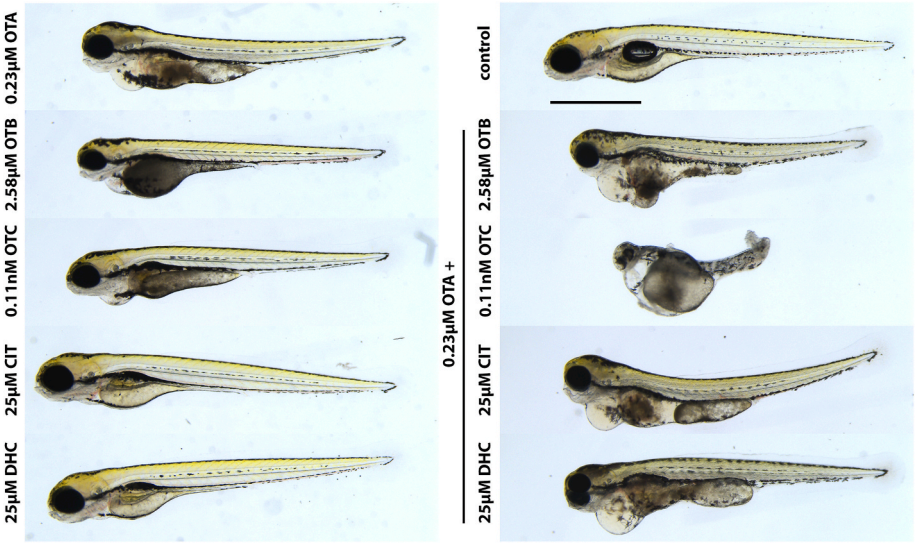

Fig. 6. Sublethal effects of mycotoxins alone (left), and the combined impacts of OTA with OTB, OTC, CIT, or DHC on 120 hpf zebrafish embryos (120 h treatment). Representative developmental defects are demonstrated (scale bar: 1 mm). The phenotypic symptoms were much more pronounced in co-exposure studies compared to the individual mycotoxin treatments.

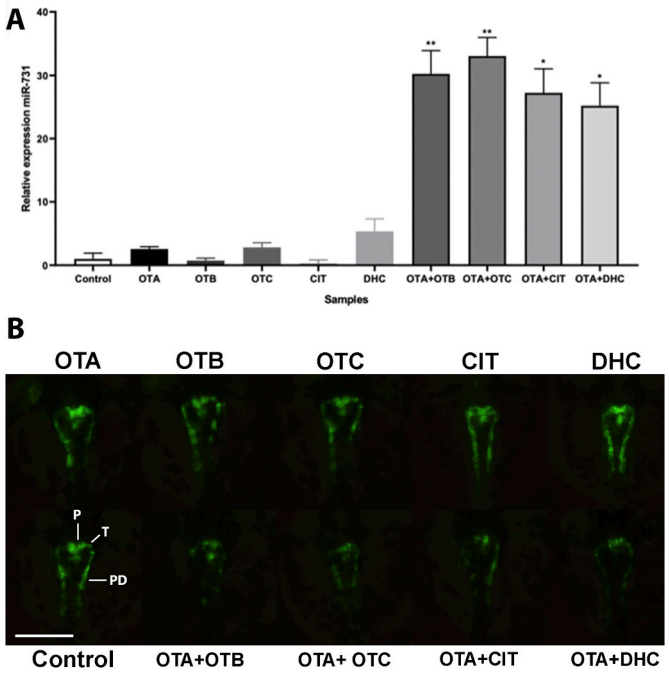

Fig. 7. Effects of individual and combined mycotoxin treatment on the expression of miR-731 (A) and the representative pronephric phenotypes in transgenic zebrafish embryos after 48 h treatment (B). OTA (0.23 μM), OTB (2.25 μM), OTC (0.055 nM), CIT (3.125 μM), and DHC (3.125 μM) did not affect significantly the expression of miRNA after exposure, while considerable increase (*$p < 0.05$, **$p < 0.01$) caused by the co-treatments (A). Pronephros of Tg(wt1b:GFP) 48 hpf transgenic embryos are demonstrated in fluorescent (GFP filter) images (B): Co-exposure resulted in phenotypic changes affecting the overall structure of the pronephros (scale bar: 250 μm; P, podocytes of renal corpuscle; T, tubule; PD, pronephric duct).

zebrafish embryos were employed. Our main observations/conclusions are the following. (1) Typically, OTC showed much higher toxicity than the parent mycotoxin OTA. (2) Even subtoxic concentrations of certain mycotoxins or mycotoxin metabolites can significantly increase the toxic impact of OTA. (3) Most of the mycotoxin pairs tested caused additive effects or synergism as their combined impacts. (4) Different models applied can show distinct (sometimes opposite) effects. (5) Mycotoxin combinations considerably increased miR-731 levels in zebrafish embryos, which is an early marker of the toxicity on kidney development. (6) In combinations, even the barely toxic mycotoxins can cause significant toxicity, thus the co-exposure to mycotoxins (and/or mycotoxin metabolites) should be seriously considered during risk assessment.

CRediT author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A. Supplementary data

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