HUNGARIAN UNIVERSITY OF
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EFFECTS OF EMBRYONIC EXPOSURE TO AFLATOXIN B1 ON ZEBRAFISH (*DANIO RERIO*) DEVELOPMENT AND INNATE IMMUNE SYSTEM

Thesis of the PhD Dissertation

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1. BACKGROUND AND OBJECTIVES

Mycotoxin contamination of foods and feeds poses a global human and animal health risk and leads to economic problems (Marroquín-Cardona et al., 2014). Aflatoxin B1 (AFB1) is one of the most prominent mycotoxins with the highest acute and chronic toxicity and is frequently found in cereals, oilseeds, spices, and their products (Filazi and Tansel, 2013). Changes in environmental and weather conditions due to climate change may favor the growth and/or toxin production of aflatoxigenic filamentous fungi. Various predictive models forecast an increase in AFB1 contamination of certain grains, especially maize, and the expansion of affected geographical regions in the near future, which can result in an elevated risk to human and domestic animal health (Battilani et al., 2016; Battilani and Leggieri, 2015; Mitchell et al., 2016).

Pregnancy or gestation is a highly vulnerable period for the consequences of AFB1 exposure, as the toxin can cross the placental barrier and potentially harm the developing embryo (Partanen et al., 2010; Wangikar et al., 2005). Embryonic exposure to environmental stress factors may lead to a broad range of adverse biological effects, from serious developmental problems — e.g., teratogenic effects — to long-term consequences such as increased susceptibility to adult diseases or reduced resistance to certain pathogens. In this regard, the role of environmental influences on the developing immune system must be highlighted. Stress during the critical period of embryonic hematopoiesis can result in autoimmune and chronic inflammatory diseases or lead to compromised immunocompetence (Goldstein et al., 2020; Holladay, 1999; Holladay & Smialowicz, 2000; Landreth, 2002; Rychlik & Sillé, 2019; Zhang et al., 2005). Therefore, it is paramount to understand the biological effects of xenobiotic exposure during critical periods of development. Although some pathological features of aflatoxicosis in certain fish, bird, and mammal species have been known for a long time, the embryotoxic properties of AFB1 need to be further explored. Low birth weight and growth faltering are the most common symptoms of human *in utero* AFB1-exposure (Lauer et al., 2019; Shuaib et al., 2010). However, the underlying biological processes are not yet completely understood.

Zebrafish shares many molecular, developmental, physiological and morphological similarities with mammals and humans, making this fish a suitable and promising model for a better understanding of xenobiotic exposure-related human diseases (Howe et al., 2013; Lieschke & Currie,

2007; Scholz et al., 2008). The development of zebrafish embryos occurs externally, which allows us to explore the embryotoxicity of AFB1 independently from maternally mediated harmful effects. The aim of this doctoral work was to provide a detailed picture of the biological, direct effects of AFB1 exposure on the zebrafish embryonic development and innate immune system at relatively low concentrations. To accomplish this goal, different biochemical, molecular biological, bioinformatical, toxicological and immunological methodologies were applied in an integrated manner across the aspects listed below.

Objectives

Characterization of the biological effects of sublethal, embryonic exposure to AFB1 as follows:

1. Detection of morphological alterations of the whole zebrafish embryos/larvae
2. Perform total RNA sequencing and transcriptome analysis in order to identify the biological pathways most prominently affected by AFB1 exposure
3. Investigation of the effects on the zebrafish embryonic innate immune system
    a. neutrophil granulocyte number and distribution in the whole embryos/larvae
    b. production of nitric oxide *in vivo*
    c. L-arginine level
    d. behavior of neutrophil granulocytes in a tail fin transection-induced local inflammation model
    e. expression of immune- and inflammation-related genes
4. Investigation of the effects on the gastrointestinal system
    a. relative length of the gut
    b. functional/anatomical alterations of the digestive tract
    c. expression of digestive system morphogenesis-associated genes
5. Investigation of the effects on the energy metabolism-related processes of the embryos/larvae
    a. yolk lipid mobilization
    b. expression of lipid transport- and metabolism-associated genes

2. MATERIAL AND METHODS

2.1. Husbandry and breeding conditions of zebrafish

Zebrafish laboratory-AB and transgenic Tg(*mpx:EGFP*) lines were maintained in a recirculation system (Tecniplast S.p.A., Italy) at the Institute of Aquaculture and Environmental Safety, Hungarian University of Agriculture and Life Sciences under the following housing conditions. Illumination: 14/10 dark/light; water temperature: 25.5 ± 0.5 °C; water conductivity: 550 ± 50 µS (system water). Fish were fed twice a day with pellet feed having an age-appropriate particle size according to the manufacturer's recommendation (SDS Zebrafeed) and once with live shrimps *(Artemia salina)*. Spawning of adult fish was carried out in specially designed breeding tanks equipped with an inserted grid and a divider. After spawning, the embryos were collected in 10 cm Petri dishes containing fresh system water.

2.2. Embryonic exposure to sublethal concentrations of aflatoxin B1

To determine the sublethal concentration range I performed a 120 hours Fish Embryo Toxicity (FET) test based on the OECD 236 guideline. Briefly, 8-19 cell stage zebrafish embryos were placed individually in 24-well cell culture plates containing 0.03125, 0.0625, 0.125, 0.25, and 0.5 mg/L AFB1 solutions (V = 2 mL; N = 2x3x10 embryo/group). Dimethyl sulfoxide (DMSO) was used as a solvent control (0.01 v/v %). The exposure lasted 120 hours post fertilization (hpf) while the survival of the embryos was continuously (daily) monitored. Next, the concentration-response curve and the 1, 10, and 50% lethal concentration (LC) values were calculated using the Origin Pro software. The applied sublethal concentrations (0.025, 0.05, 0.075, 0.1 mg/L) during further experiments were determined on the basis of the LC10 value.

2.3. Evaluation of morphological alterations

At the end of embryonic exposure to AFB1 (120 hpf) the larvae were anesthetized in tricaine methanesulfonate solution (MS222, 168 mg/L), positioned laterally and imaged under a stereo microscope (Leica M205 FA, Leica DFC7000T camera, Leica ApplicationSuiteX software, Leica Microsystems GmbH, Germany) (N=3x30/group). Measurements of total

body length, swim bladder area and the relative length of the gut (distance between the esophagus and the anus) were performed from the pictures of the larvae using ImageJ software (N = 3x30 larvae/group).

2.4. RNA sequencing and transcriptome analysis

Total RNA sequencing and analysis were performed at the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen. After embryonic exposure to 0.1 mg/L AFB1 (the highest (≈LC10) value of the selected sublethal concentrations) the larvae were transferred into chilled microcentrifuge tubes containing 200 µL of Trizol reagent and homogenized (N=4x30 larvae/group). RNA isolation was performed with chloroform/isopropanol extraction and ethanol precipitation. The integrity of the isolated RNA was checked using the Eukaryotic Total RNA Nano Kit on an automatic gel electrophoresis apparatus Agilent BioAnalyzer. The RNA library was created by the Ultra II RNA Sample Prep Kit. Poly-A RNAs were captured by oligo-dT magnetic beads followed by elution and fragmentation (94 °C). Complementary DNAs (cDNAs) were generated using randomly primed reverse transcription-polymerase chain reaction. After adapter ligation the sequencing run was executed on an Illumina NextSeq500 instrument. Raw sequencing data were aligned to the zebrafish reference genome (*Danio rerio*, GRCz11) using a HISAT2 algorithm. Differentially expressed genes were identified by a DESeq1 algorithm and the StrandNGS software. The biological processes significantly altered by the AFB1 treatment were evaluated by Gene Ontology Enrichment Analysis (GOEA) using the Cytoscape/ClueGo software (Bindea et al., 2009).

2.5. Determination of gene expression by RT-qPCR

Expression of different marker genes selected on the basis of transcriptome analysis was measured by reverse transcriptase polymerase chain reaction (RT-qPCR). At the end of the AFB1 exposure the larvae were transferred into chilled microcentrifuge tubes containing 200 µL of Trizol reagent and homogenized (N=5x30 larvae/group). RNA isolation was performed with chloroform/isopropanol extraction and ethanol precipitation. The quality and quantity of isolated RNA were determined using NanoDrop One spectrophotometer. cDNA was generated using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). PCR reactions were performed with the 5x HOT FIREPol EvaGreen qPCR Supermix on Light

Cycler 480II instrument. Expression levels of the target genes were normalized to the expression of the *ef1a* housekeeping gene.

2.6. Determination of neutrophil granulocyte cell number and distribution

To quantify neutrophil granulocyte number and evaluate their distribution in the embryos/larvae a transgenic Tg(mpx:EGFP) zebrafish reporter line was used. Neutrophil granulocyte number (frequency of EGFP+ cells) was measured at the Department of Immunology, Faculty of Medicine, University of Debrecen by fluorescence-activated cell sorting (FACS) and analysis. After the AFB1-exposure the larvae were placed in chilled microcentrifuge tubes, rinsed in Ringer solution, then dissociated in 0.25% Tripsin-EDTA (N=2x3x15 larvae/group). The digestion process was stopped by adding fetal bovine serum (FBS) and calcium chloride to the samples. The samples were centrifugated (400 g; 5 min), then resuspended with 5% FBS/PBS and filtered through 40 µm pore sized cell strainers. The frequency of EGFP+ cells in each sample was determined per 10^5 cells using a NovoCyte Flow Cytometer.

Distribution of neutrophil granulocytes in the whole larvae were assessed after imaging the anesthetized (MS-222, 168 mg/L), laterally positioned larvae under a fluorescence stereo microscope (Leica M205 FA, Leica DFC7000T camera, Leica Application Suite X software, Leica Microsystems GmbH, Germany) equipped with a GFP2 filter. The accumulation of EGFP+ cells in the yolk and intestinal area were quantified by the ImageJ software.

2.7. *In vivo* detection of nitric-oxide production

Nitric-oxide (as an inflammatory mediator) production in the zebrafish larvae was measured by a fluorescence probe, diaminofluorescein-FM diacetate (DAF-FM-DA). At the end of AFB1-exposure the larvae were placed individually in 96-well cell culture plates containing 200 µL of 5 µM DAF-FM-DA solution and incubated for 1 hour at 25.5 °C in the dark. Next, the larvae were rinsed in system water, then lateral-view images were taken under a fluorescence stereo microscope (Leica M205 FA, Leica DFC7000T camera, Leica Application Suite X software, Leica Microsystems GmbH, Germany) equipped with a GFP2 filter. Nitric-oxide level was determined by measuring fluorescence intensities of the yolk and

intestinal area and the whole larvae using the ImageJ software (N = 2x10 larvae/group).

2.8. Amino acid analysis

L-arginine level was measured by UHPLC. Briefly, after AFB1-exposure the larvae were transferred into microcentrifuge tubes, their wet weight was measured, then the larvae were homogenized in SDS-containing borate buffer (N=3x30 larvae/group), centrifuged (20000 g, 10 min), filtered through a 3kDa Pall filter, and dried in a vacuum concentrator (SpeedVac, Thermo Scientific). Samples were derivatized with an AccQTag Ultra Kit, examined in duplicates on a UPLC (H-Class, Waters) and analyzed using the Empower software.

2.9. Tail fin transection model

The tail fin transection was performed on a neutrophil-specific transgenic Tg(mpx:EGFP) zebrafish reporter line which allows us to monitor *in vivo* the neutrophil granulocyte recruitment at the site of injury. After AFB1 exposure the tail fin of the anesthetized (MS-222, 168 mg/L) larvae were transected with a sterile razor blade under a stereo microscope. Next, the larvae were transferred individually into 24-well cell culture plates containing fresh system water. Then, lateral view images of the larvae were taken under a fluorescence stereo microscope (Leica M205 FA, Leica DFC7000T camera, Leica Application Suite X software, Leica Microsystems GmbH, Germany) equipped with a GFP2 filter. The number of neutrophils at the site of injury was determined by manual counting using ImageJ software (N=5x10 larvae/group).

2.10. Evaluation of yolk lipid mobilization

Effects of the AFB1 exposure on the yolk lipid content were determined by the Oil-Red-O (ORO) staining method. At the end of the AFB1 exposure, the anesthetized (chilled water) larvae were rinsed with PBS and fixed in 4% paraformaldehyde at 4 °C for 6 hours. After fixation, the larvae were incubated for 2 hours in microcentrifuge tubes containing 0.1% ORO-60% isopropanol solution. Next, the stained larvae were rinsed with PBS, transferred into 95% glycerol, then bright field images were taken in front of a uniformly lit (white) background under a stereo microscope (Leica M205 FA) (N=2x10 larvae/group). Yolk lipid content was assessed by

measuring optical density of the yolk of the laterally positioned larvae using the ImageJ software following "Rodbard-calibration" (https://imagej.nih.gov/ij/docs/examples/calibration/).

2.11. Fluorescence microbead assay

We performed the fluorescence microbead assay in order to evaluate the functional-anatomical alterations of the gastrointestinal system. Briefly, carboxylate-modified polystyrene latex beads (2 μm, fluorescent red, Sigma-Aldrich) were washed with reverse-osmosis water, centrifuged and re-suspended (2.5 %). After AFB1-exposure the larvae were transferred into microcentrifuge tubes containing 0.025% microbead suspension and incubated for 3 hours at 25.5 °C (the tubes were left open). After incubation, the larvae were rinsed thoroughly with fresh system water, anesthetized (MS-222, 168 mg/L), positioned laterally and imaged under a fluorescence stereo microscope (Leica M205 FA, Leica DFC7000T camera, Leica Application Suite X software, Leica Microsystems GmbH, Germany) equipped with mCherry filter. Microbead accumulation in the pharyngeal and intestinal region of the larvae were quantified by measuring fluorescence intensities using the ImageJ software.

2.12. Statistical analysis

Statistical data were calculated after the Shapiro-Wilk normality test by one-way parametric ANOVA and post-hoc Dunnett test or Kruskall-Wallis test and post-hoc Dunn test using the GraphPad Prism 8 software. Differentially expressed genes were identified using Moderated T-test with the Benjamini-Hochberg procedure. Results are presented as the mean ±standard deviation (SD).

3. RESULTS

3.1. Morphological and transcriptional changes in zebrafish caused by sublethal, embryonic exposure to AFB1

The sublethal exposure to AFB1 did not result in dramatic morphological alterations of the zebrafish embryos/larvae, however a slight but significant decrease could be detected in the total body length at 0.1 mg/L and a more obvious decrease could be observed in the swim bladder area in a concentration-dependent manner.

Total RNA sequencing and analysis identified 1216 significantly differentially expressed genes at 0.1 mg/L. Among these genes 829 were down-regulated and 387 were up-regulated. The Gene Ontology Enrichment analysis revealed several biological pathways affected by the AFB1-exposure. Among the up-regulated genes, immune response- and inflammation-associated gene sets were significantly enriched. In contrast, oxidoreductase activity and energy metabolism, e.g. lipid and organic acid metabolism were the most prominently repressed biological processes.

3.2. Effects of AFB1 on the embryonic innate immune system

Based on the transcriptomic results, next, this work has focused on the immune-modulatory and inflammatory effects of the sublethal AFB1 exposure in the zebrafish embryos/larvae. As expected, the selected immune response-associated genes (il1β, mmp9, cxcl8b.1, cxcl18b) were significantly up-regulated in the AFB1-exposed groups in a concentration-dependent manner – especially in the case of two neutrophil chemoattractant factors (cxcl8b.1, cxcl18b) – which were consistent with the results of our transcriptome analysis.

Images of the AFB1-exposed 120 hpf transgenic larvae showed a widespread diffuse distribution of the neutrophil granulocytes in their whole body. In addition, an increased neutrophil accumulation could be observed in the yolk and intestinal area, which is a well-defined abdominal region of the larvae. Thus, the number of neutrophils was quantified at this region and showed a strong increase at 0.025−0.075 mg/L. Besides, the FACS-determined frequency of the EGFP+ cells in the whole larvae decreased significantly, but only at the highest applied concentration (0.1 mg/L).

In the tail fin transection model the neutrophil granulocyte number significantly decreased at the site of injury in the AFB1-exposed groups in a concentration-dependent manner. This has raised the possibility of a vascular damage may have caused by the toxin and its contribution to the impaired neutrophil recruitment. However, no obvious differences were observed in the vascular morphology of the larvae between the control and treatment groups.

3.3. Effects of AFB1 on nitric oxide production and L-arginine level

After the AFB1 exposure, an elevated nitric oxide generation was observed in the 120 hpf larvae. Fluorescence intensity proportional to nitric oxide level was significantly increased in the whole larvae in a concentration-dependent manner. Moreover, this increased production was prominent in the yolk and intestinal area, similar to the pattern shown by the neutrophil granulocyte accumulation. The elevated nitric oxide production was consistent with a significant decrease in the nitric oxide synthase substrate, L-arginine level.

3.4. Effects of AFB1 on the gastrointestinal system

As the increased neutrophil granulocyte accumulation and nitric oxide production indicated, the yolk and intestinal region of the zebrafish embryos/larvae were significantly affected by the AFB1 exposure. Besides, microscope images of the 120 hpf larvae showed visible signs of underdevelopment of the intestinal system. In addition, several differentially expressed, digestive system morphogenesis-associated genes (clda; aldh1a2; dhrs9; pllp; cdx1b) were identified by our RNA-seq. As expected, the two selected marker genes (dhrs9, cdx1b) for RT-qPCR were significantly down-regulated in the AFB1 exposed groups at 0.05, 0.075 and 0.1 mg/L.

To explore further the gastrointestinal damage caused by AFB1, first, relative gut length was measured, and it showed a significant decrease even at the lowest applied concentration (0.025 mg/L) compared to the control. Next, the fluorescent microbead assay was applied in order to evaluate functional/anatomical alterations of the digestive tract. Microbead

accumulation dramatically decreased in the intestinal bulb, mid-gut and distal gut of the larvae after AFB1 exposure. In contrast, parallel to the reduced quantities of fluorescent beads in the gut, a strong increase could be detected in the oral cavity/pharyngeal region of the larvae at 0.075 and 0.1 mg/L AFB1.

3.5. Effects of AFB1 on yolk lipid mobilization

To get a deeper insight into the disruption of lipid metabolism and utilization, first, Gene Set Enrichment Analysis (GSEA) of our RNA Seq data was performed using a yolk lipid metabolism- and transport-associated gene set identified by Fraher et al (2016). Results of the GSEA demonstrated significant enrichment of these genes in the AFB1 exposed groups. (False Discovery Rate, FDR < 0.1; Normalized Enrichment Score, NES: -1.53). Next, four marker genes from this set (mtp, apoa4, apobb.1, fabp1b.1) were selected for RT-qPCR. Expression level of mtp, apoa4a and apobb.1 showed a slight decline at 0.025 mg/L and a more obvious, significant decrease at 0.05, 0.075, and 0.1 mg/L AFB1. In addition, the expression level of fabp1b.1 decreased significantly even at the lowest applied concentration (0.025 mg/L).

Oil Red O staining of the whole larvae allowed us to quantify the alterations of lipid content and distribution and evaluate yolk lipid mobilization. Consistent with the results of our molecular-biological analyses, optical densities of the abdominal region (yolk and intestinal area of the larvae) showed a significant, concentration-dependent increase in the treatment groups indicating an AFB1-induced defective yolk lipid mobilization.

4. NEW SCIENTIFIC RESULTS

1. The results demonstrated significant, global transcriptional changes manifested by induced immune and inflammatory response-related and repressed energy metabolism-related pathways in the 120 hpf zebrafish larvae after sublethal, embryonic exposure to aflatoxin B1 (AFB1).

2. The results demonstrated altered neutrophil granulocyte number and distribution, as well as impaired neutrophil granulocyte response to a local injury in the 120 hpf zebrafish larvae after sublethal, embryonic exposure to AFB1.

3. The results demonstrated an elevated nitric oxide production and depleted L-arginine level in the 120 hpf zebrafish larvae after sublethal, embryonic exposure to AFB1.

4. The results demonstrated a defective yolk lipid mobilization in the 120 hpf zebrafish larvae after sublethal, embryonic exposure to AFB1.

5. The results demonstrated abnormal gastrointestinal development in the 120 hpf zebrafish larvae after sublethal, embryonic exposure to AFB1.

5. CONCLUSIONS AND PERSPECTIVES

Aflatoxin B1 (AFB1) is one of the most prominent mycotoxins and a considerable risk to human and domesticated animal health. Although its embryotoxic potential has been known since the 1960s, the biological effects of embryonic exposure to AFB1 especially on mammals and humans are not completely understood yet. One of the most important suspected contributing factors to AFB1-induced adverse pre- and postnatal outcomes is the elevated production of pro-inflammatory mediators. Thus, of prime importance in the investigation of the embryonic effects of AFB1 are the immunomodulatory and inflammatory potential.

Related studies using zebrafish have focused mostly on the neurotoxic and hepatotoxic effects of the toxin (Park et al., 2020; Wu et al., 2019; Zhou et al., 2017; Zuberi et al., 2019). Lethal concentration values determined by these studies are consistent with the mortalities detected during the experiments of this doctoral work. However, it must be emphasized that the durations of AFB1 exposure in the aforementioned studies were different from ours.

Sublethal embryonic exposure to AFB1 did not result in dramatic morphological changes in the zebrafish larvae. A similar phenomenon was observed by Wu et al. (2019). Nevertheless, more significant alterations could be detected at the transcriptional level. AFB1 exposure significantly affected the expression of more than 1200 genes identified by our RNA-Seq. Among the down-regulated differentially expressed genes, oxidoreductase, and monooxygenase activity- and energy metabolism-associated gene sets were significantly over-represented. Oxidoreductase enzymes (e.g., superoxide dismutase, glutathione peroxidase, NADPH quinone reductase) play a crucial role in the elimination of reactive oxygen species (ROS) (Battelli et al., 2016; Ngoka, 2008). Our transcriptome analysis identified 147 down-regulated, oxidoreductase activity-related genes in the AFB1-exposed groups. These results can confirm the role of AFB1 in oxidative stress induction, which is frequently reported by different toxicological studies.

Immune response and inflammation-associated gene ontology terms were predominantly enriched among the AFB1-induced, differentially expressed genes. These transcriptional changes related to the innate immune system

were accompanied by an altered neutrophil granulocyte distribution and elevated nitric oxide production in the AFB1-exposed larvae, especially in the yolk and intestinal area. Similarly, Dey & Kang (2020) observed a strong ROS-induction at this region in the AFB1-exposed 6 dpf zebrafish larvae. Besides, as expected, the increased nitric-oxide generation was coupled with L-arginine depletion. Taken together, these results indicated an AFB1 exposure-induced systemic-like inflammation in the developing zebrafish embryos/larvae. Next, a tail fin transection model was applied in order to evaluate the response of the innate immune system of the AFB1-exposed larvae to a local injury. In this model an impaired neutrophil recruitment was observed as revealed by a concentration-dependent decrease of neutrophil number at the site of injury at different times. Although the frequency of these innate immune cells in the whole larvae differed from the control only at the highest applied concentration (0.1 mg/L), the impaired recruitment has already been detected in the lower concentration groups. In addition, no significant alteration could be observed in the vascular system of the larvae. Several studies have described a modulation of neutrophil granulocyte response in zebrafish by different xenobiotics, e.g., famoxadone-cymoxanil (Cheng et al., 2020) or silver nanoparticles (Chen et al., 2021). Taken together, the embryonic exposure to sublethal concentrations of AFB1 induced pro-inflammatory responses and modulated the innate immune system of the 120 hpf zebrafish larvae.

The yolk and intestinal area of the developing embryos and larvae proved to be a prominently affected region in the AFB1-induced inflammation. For zebrafish embryos, the yolk serves as not just a storage, but a metabolically active system with important transport processes (Fraher et al., 2016). Park et al. (2020) detected significantly higher yolk sac diameter in the AFB1-exposed zebrafish embryos compared to the control. Besides, this doctoral work revealed a strong repression of several lipid metabolism- and transport-related genes after the toxin exposure. These results raised the possibility of an AFB1-induced defective yolk lipid mobilization. As we expected, the yolk lipid content of the AFB1-exposed larvae increased significantly in a concentration-dependent manner, which reflects the impaired ability to yolk lipid mobilization. Even though the toxicity of aflatoxins has been widely investigated, there is little information available about the adverse effects of embryonic AFB1-exposition on the lipid metabolism and utilization. Some research studies

reported disturbed lipid metabolism after the analysis of blood and liver samples from AFB1-treated adult rats (Rotimi et al., 2017; Ugbaja et al., 2020). It is also important to emphasize that the abnormal yolk lipid "accumulation" in the zebrafish larvae was associated with inflammation-induction. However, more detailed exploratory work is needed to draw conclusions about the causal relationship between the elevated lipid content and inflammation in the toxicity of embryonic AFB1-exposure.

Although previous toxicological studies have shown that AFB1 can cause different gastrointestinal alterations in rats, mice, and broiler chickens, the effects of embryonic AFB1-exposure on the digestive tract and the possible underlying mechanism are still poorly understood. The results of this doctoral work demonstrate that the AFB1 exposure during the zebrafish embryonic development can cause significant alterations in the gastrointestinal system manifested by repression of digestive tract morphogenesis-associated gene set and anatomically/functionally underdeveloped intestinal system. Similarly, this phenomenon was also associated with an increased neutrophil granulocyte accumulation and nitric oxide production. However, the role of inflammation in AFB1-induced gastrointestinal alterations needs to be further explored.

The biological consequences of embryonic exposure to AFB1 have not been fully characterized. Based on the results of previous in vitro tests and experiments utilizing various vertebrate models, as well as of the human surveys and case studies, it can be assumed that the pro-inflammatory and immunomodulatory effects, the alterations of the gastrointestinal system and the disturbance of energy metabolism may significantly contribute to the AFB1-associated adverse birth outcomes, growth faltering, and postnatal immunological problems (Smith et al., 2017). The present doctoral work has aimed to characterize the direct embryotoxic effects of AFB1 at sublethal concentrations in a comprehensive manner using zebrafish as a widespread model system in toxicological and pharmacological research and for the study of human diseases. The applied morphological, transcriptional, immunological and toxicological examinations provided consistent results. In summary, the embryonic exposure to relatively low, sublethal concentrations of AFB1 significantly affected the innate immune system, the energy metabolism, and the gastrointestinal system of the developing zebrafish embryos/larvae, which can strongly determine the later-life outcomes of the individuals. Zebrafish shares common molecular mechanism and pathways with

mammalian inflammatory responses, gastrointestinal development, and energy metabolism (Flynn III et al., 2009; Forn-Cuní et al., 2017; Marza et al., 2005; Miyares et al., 2014; Quinlivan & Farber, 2017; Wallace & Pack, 2003). However, since the zebrafish embryo model does not provide the mother-placenta-fetus system, extrapolation of our results to mammals and humans should be precautious.

Suggestions for further research

a) Further investigation of immunomodulatory effects of embryonic AFB1-exposure using macrophage-specific transgenic zebrafish reporter line.

b) Exploring the effects of embryonic AFB1-exposure on tissue regeneration in a tail fin transection zebrafish model.

c) Histopathological investigation of gastrointestinal alterations.

d) Exploring long-term effects of embryonic AFB1-exposure by monitoring juvenile and adult survival, assessing the immunocompetence of adults and detecting transgenerational changes.

e) Using the methodologies and endpoints applied in this doctoral work to test protective or alleviative effects of compounds in the toxicity of AFB1.

6. PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

6.1. Publications in scientific journals

Ivanovics, B., Gazsi, G., Reining, M., Berta, I., Poliska, S., Toth, M., ... & Czimmerer, Z. (2021). Embryonic exposure to low concentrations of aflatoxin B1 triggers global transcriptomic changes, defective yolk lipid mobilization, abnormal gastrointestinal tract development and inflammation in zebrafish. Journal of Hazardous Materials, 416, 125788. https://doi.org/10.1016/j.jhazmat.2021.125788

Gazsi, G., Czimmerer, Z., **Iványovics, B.**, Berta, I. R., Urbányi, B., Csenki-Bakos, Z., & Ács, A. (2021). Physiological, developmental, and biomarker responses of zebrafish embryos to sub-lethal exposure of bendiocarb. Water, 13(2), 204. https://doi.org/10.3390/w13020204

Ács, A., Liang, X., Bock, I., Griffitts, J., **Iványovics, B.**, Vásárhelyi, E., ... & Csenki, Z. (2022). Chronic Effects of Carbamazepine, Progesterone and Their Mixtures at Environmentally Relevant Concentrations on Biochemical Markers of Zebrafish (Danio rerio). Antioxidants, 11(9), 1776. https://doi.org/10.3390/antiox11091776

Liang, X., Csenki, Z., **Iványovics, B.**, Bock, I., Csorbai, B., Molnár, J., ... & Ács, A. (2022). Biochemical Marker Assessment of Chronic Carbamazepine Exposure at Environmentally Relevant Concentrations in Juvenile Common Carp (Cyprinus carpio). Antioxidants, 11(6), 1136. https://doi.org/10.3390/antiox11061136

Iványovics, B. (2017). A mikotoxinok, mint halegészségügyi kockázati tényezők. HALÁSZAT - TUDOMÁNY 3:2, pp. 3-7. Paper: HU ISSN 0133-1922

6.2. Oral presentations related to the topic of the dissertation

Iványovics, B., Gazsi, Gy., Póliska, S., Tóth, M., Csenki-Bakos, Z., Berta, I., Reining, M., Ács, A., Domokos, A., Bacsi, A. … & Czimmerer, Z. (2022). Characterization of non-lethal aflatoxin B1 exposure-induced inflammation and immune modulation in zebrafish embryos In: Tóthová, M., Lidiková, J., Candráková, K., Hollý, D., Mihaľ, M. (Eds.). Scientific Conference of PhD Students of FAFR, FBFS and FHLE SUA in Nitra with international participation, Nitra, p. 49.

Iványovics, B., Gazsi, Gy., Ács, A., Csenki-Bakos, Zs., Reining, M., Sulyok, Z., Urbányi, B., Czimmerer, Zs. (2017). Xenobiotikumok gyulladásos folyamatokra gyakorolt hatásainak vizsgálati lehetőségei zebradánió (Danio rerio) modellrendszeren In: Darvas, Béla; Bakonyi, Gábor; Székács, András; Szoboszlay, Sándor (szerk.) VII. Ökotoxikológiai konferencia Budapest, Magyarország, Magyar Ökotoxikológiai Társaság pp. 18-19.

Iványovics, B., Gazsi, Gy., Reining, M., Ács, A., Csenki-Bakos, Zs., Berta, I., Urbányi, B., Czimmerer, Z. (2019). Embrionális aflatoxin B1-expozíció gyulladásos folyamatokra gyakorolt hatásainak vizsgálata zebradánión In: Magyar, Toxikológusok Társasága (szerk.) TOX'2019 Tudományos Konferencia, Program, kivonatok 132 p. Paper: C2-5

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6.3. Poster presentations related to the topic of the dissertation

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7. PUBLICATIONS NOT RELATED DIRECTLY TO THE TOPIC OF THE DISSERTATION

7.1. Publications in scientific journals

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