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Larval zebrafish as an *in vitro* model for evaluating toxicological effects of mycotoxins.

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Abstract

The presence of mycotoxins in food has created concern. Mycotoxin prevalence in our environment has changed in the last few years maybe due to climatic and other environmental changes. Evidence has emerged from *in vitro* and *in vivo* models: some mycotoxins have been found to be potentially carcinogenic, embryogenically harmful, teratogenic, and to generate nephrotoxicity. The risk assessment of exposures to mycotoxins at early life stages became mandatory. In this regard, the effects of toxic compounds on zebrafish have been widely studied, and more recently, mycotoxins have been tested with respect to their effects on developmental and teratogenic effects in this model system, which offers several advantages as it is an inexpensive and an accessible vertebrate model to study developmental toxicity. External post-fertilization and quick maturation make it sensitive to environmental effects and facilitate the detection of endpoints such as morphological deformities, time of hatching, and behavioral responses. Therefore, there is a potential for larval zebrafish to provide new insights into the toxicological effects of mycotoxins. We provide an overview of recent mycotoxin toxicological research in zebrafish embryos and larvae, highlighting its usefulness to toxicology and discuss the strengths and limitations of this model system.

Keywords

zebrafish; larvae; embryos; mycotoxins; mixtures; toxicology; model system; embryonic development

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Declaration of interests

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1. Introduction

The presence of mycotoxins in the food chain has been reported in several publications (Stanciu et al., 2017; Marijani et al., 2019; Juan et al., 2017, 2019). In fact, it constitutes one of the most important aspects involved in spoilage of food and also in feed (Tirado et al., 2010). Mycotoxins are secondary metabolites produced by filamentous fungi (molds), such as *Aspergillus spp.*, *Penicillium spp.*, and *Fusarium spp.*, in the matrix where they grow (food and feed) and can appear during growing, harvesting, storage and processing. They probably perform ecological functions against other organisms and protect fungi from oxidative stress; however, the main importance of these compounds is the toxicological chronic effects that can cause in humans and animals and acute toxicity which has occurred very occasionally (Zain 2011). There are approximately 400 compounds currently recognized as mycotoxins, although only few of them are addressed by food legislation. The qualitative and quantitative mycotoxin profile which a mold produces on a food commodity depends on the ecological and processing parameters of the particular foodstuff and the fungi *spp.* (Tirado et al., 2010). Mycotoxins are classified into different groups depending on their molecular structure, producer fungus, and toxicity (Juan-García et al., 2013). Figure 1 collects the chemical structure of some mycotoxins.

Mycotoxin contamination in food and feed includes coffee, apples, tomato, strawberries, grain cereals, dried fruits and a big etc. where geographic origin, post-harvest processes and storage times play an important role. In general, food contains many naturally occurring chemicals, of different origin and from different source and combined exposure to multiple chemical is also of great interest (Agahi et al., 2020; Juan-García et al., 2018, 2019a, 2019b, 2020) as there are limitations in knowledge and scientific evidence regarding potential harm in humans, animals and environment as well as the risk assessment of mixtures of chemical exposures (EFSA, 2019). The importance of mycotoxins detection in food, which will be consumed by humans and animals is to understand and determine how mycotoxins will affect cell mechanisms. So that, the development of methods and the use of biological models that hit to study pathways implicated in the toxic effect of mycotoxins is necessary and nowadays becoming crucial for mycotoxins legislation (Juan-García et al., 2013; EFSA, 2019).

World Health Organization (WHO) reports that acute and chronic effects of mycotoxins on humans and animals (especially monogastrics) depends on species and susceptibility of an animal within a species. There are more cases of acute toxicity for animals than for humans although control of mycotoxins for food and feed is mandatory for some of them, as set in legislation and followed by authorities. The most important acute effect reported for mycotoxins have been aflatoxicosis (due to high doses of aflatoxins) and associated in producing damage to the liver (WHO, 2018). Nonetheless, chronic effects as genotoxicity, hepatotoxicity, nephrotoxicity, DNA damage, cancer in several organs (liver, kidney, spleen), effects in fetal development and on the immune system, gastrointestinal disturbances, nausea, abdominal pain, vomiting, skin irritation, intestinal irritation, diarrhea, alterations in bone marrow, etc. have been associated several mycotoxins (Zain 2011).

The effects of these natural food contaminants on human health are known and have been classified by the International Agency for Research in Cancer (IARC) (IARC, 1993) for chronic exposure. Several *in vitro* studies have suggested the capacity of mycotoxins to alter mitochondrial membrane potential, cell cycle distribution, cell death, production of reactive oxygen species (ROS). These assays have been performed in different cell lines, e.g., in Caco-2 cells (Ferrer et al., 2008; Prosperini et al., 2013a, 2013b), in HepG2 cells (Juan-García et al., 2019), in CHO-K1 cells (Mallebrera et al., 2015), in murine monocyte macrophage (RAW 264.7) (Dornetshuber et al., 2007), in human non-small cell lung cancer (A549) cells (Gammelsrud et al., 2012). Other mycotoxins have been studied, e.g., enniatins (ENs), beauvericin (BEA), patulin (PAT), zearalenone (ZEA), T-2 toxin, and ochratoxin A (OTA). The main limitations of *in vitro* models include the lack of interactions with other cells, lack of hormonal or immunological influences, and the levels of gene expression involved in the response to mycotoxin exposure (Ghallab et al., 2014). The translation of *in vitro* data into meaningful *in vivo* effects remains unknown. Alternative model systems, e.g., animal models, have been developed and their results are promising (Csenki et al., 2019; Zhou et al., 2017). The Organization for Economic Co-operation and Development (OECD) guidelines propose the zebrafish model (i.e., *Danio rerio*) as a good model system to study ecotoxicology and neurodevelopmental toxicity (OECD 236, 2013). The properties of this model are consistent with the principle of 3Rs (i.e., Replacement, Reduction, Refinement): there is an increasing demand for alternative model systems for which the number and the suffering of experimental animals can be minimized (Novak et al., 2020). The zebrafish is an omnivorous small species easy to keep and readily reproducing in the laboratory and has transparent embryos which is suited for high-throughput toxicological studies examining developmental and neuro behavioral toxicity. The transparency of zebrafish embryos allows the application of high-resolution imaging techniques at all stages of development. Zebrafish embryos up to five days post fertilization (dpf) are considered as non-protected life stages by the EU Directive 2010/63/EU also by the Institutional Animal Care and Use Committee (IACUC) in the United States; hence zebrafish embryos represent replacement method for testing adult animals (Strähle et al., 2012; Lawrence et al., 2009).

Zebrafish has also been used to measure estrogen receptor activation *in vivo*, e.g., Competitive Estrogen Receptor Binding Assay, OECD-TG-455, 457, 493 (OECD, 2012). Zebrafish can be used in toxicology to study hepatotoxicity, neurotoxicity, and cardiotoxicity due to the property of transparent embryos, which makes it easier to observe the embryogenetic process than in mammal fetus (Hill et al., 2005; Reimers et al., 2006).

The zebrafish model correlates fairly well with the results from other vertebrate model systems (Ablain and Zon, 2013; Forn-Cuní et al., 2017), and from cell lines (Rainieri et al., 2017). Chemical structure of mycotoxins is highly diverse (Juan-García et al., 2013), therefore, specific toxicological effects cannot be estimated on the basis of molecular structure and chemical interactions.

Neuronal differentiation into mature neurons is comparable between humans and zebrafish (Troppe et al., 2003). Although the neurotransmitter system evolves faster in zebrafish than in humans, it is detected similarly at 48 hpf in zebrafish and at week 13 in humans (Semple et al., 2013 and Jeong et al., 2008). The formation, differentiation, and connectivity of

neurons require molecular mechanism that appear to be conserved in all vertebrates. We review some of the current literature on the toxicological effects associated with mycotoxin exposure in the early stages of larval zebrafish.

2. Observational changes in early stages and development in zebrafish

Zebrafish are small and transparent as embryos, high spawning rate, develop relatively rapidly, and induce a fairly low cost to raise. Such characteristics make zebrafish suitable to assess embryonic development (OECD, 2013), especially because embryogenesis is non-placental and external to the mother. Adverse endpoints, e.g., coagulation, missing heartbeat, tail detachment, developmental abnormalities such as yolk sac, pericardial edema, spinal deformity, abnormal pigmentation, fin and tail malformations, hatching, incomplete development of the head and eyes are good candidates to evaluate. Several outcomes or live index can also be considered, e.g., heart beats, blood flow, blood coagulation as well as somatic indices such as body weight, total length, gonadal and hepatic changes.

Evaluating development at different time windows is an endpoint to consider: at 1 dpf nervous system is formed, at 3 dpf the blood brain barrier is observed, after 20–24 hpf the neurotransmitter systems are detected and are almost complete at 5 dpf, and for this last time point, a broad range of behaviors is also noticeable (Jeong et al. 2008, Rico et al. 2011; Horzmann et al., 2016; Schneider et al., 2017).

3. Developmental toxicity of mycotoxins in zebrafish

To have developmental effects, a mycotoxin must be present in food commodities (Juan et al., 2017b, 2019; Oueslati et al., 2018; Stanciu et al., 2018), and in a sufficient amount to induce a particular acute defect in an embryo/larvae during a critical development point or to have a chronical exposure during the development (Juan-García et al., 2019a, 2019b; Klaric et al., 2006; Alassane-Kpembi et al., 2015). A non-exhaustive list of studies examining the effects of mycotoxin exposure in early zebrafish developmental stages are summarized in Table 1. To assess the effects of mycotoxin exposure in early stages of zebrafish, substances are dissolved in water at the desired concentration; however, it is sometimes difficult to quantify, and in this case, egg microinjection can be used to measure effects at such level (Wu et al. 2013, 2016; Khezri et al. 2018).

Mycotoxins are dissolved in water in the early stages of the zebrafish, thus, the main pathway of absorption occurs by inhalation, by gills (early evolved), or during early stages through the chorion (Martins et al. 2018; Felix et al. 2016; Sanden et al., 2012). Despite this, the concentrations used in zebrafish might not be higher than the established by OECD 236 for testing compounds (OECD, 2013).

Microinjection technique, mentioned above, enables to administrate the exact amount of the substance in the yolk sac but without overpassing 10% lethality as maximum according to the OCDE guideline for fish embryo test (OECD 236, 2013). Moreover, it is important to consider the period of exposure after embryo microinjection, since enlarging such period may compromise the assay and fall under animal testing regulations as mycotoxin might remain unabsorbed after about 7dpf (165 ± 12 hpf), which corresponds to the time that

zebrafish embryos consume their yolk sac completely (Litvak et al., 2003). With microinjection, it is easy to determine the potential capacity of a strain to degrade the toxin and also to select the safest environmental *spp*. Studies have assayed the embryotoxicity effect of deoxynivalenol (DON) (Khezri et al., 2018) and the biotransformation of ochratoxin A (OTA) (Csenki et al., 2019). By using microinjection in embryos, it is easy to study the effect of organic substances, which helps to eliminate the hypoxia conditions. It is a useful technique to investigate the toxin detoxification-properties of microbial strains.

Literature reveals that some mycotoxin exposure is associated with brain disorders as autism, suggesting that mycotoxins are potential stressors of the central nervous system either directly or through the gut-brain axis; a complex bidirectional cross-talk between the enteric and central nervous system that acts through neuroendocrine and neuroimmune signals (Muthulakshmi et al., 2018; Juan-García et al., 2019a). The presence of elevated mycotoxins (i.e., deoxynivalenol, de-epoxydeoxynivalenol, aflatoxin M1, ochratoxin A and fumonisin B1) has been reported in body fluids of autistic children (e.g., urine and serum); however, the involved biological mechanisms are unknown (Carter et al., 2019; De Santis et al., 2017a; De Santis et al., 2017b). Several *in vitro* assays have highlighted the influence of mycotoxins in the modulation of neurodevelopmental processes (Fatemi et al. 2005, 2008; Cordeiro et al. 2015), which may result in neurotoxic damage in the immature brain and in long-term behavioral effects (de Santis et al., 2019). Genes implicated in autism are associated with mycotoxins as demonstrated in the pathogen interactomes which possess or secrete toxins whose effects on genes are catalogued at the Comparative Toxicogenomics database (Davis et al., 2015).

As reported in the literature, the most common mycotoxins present in food (e.g., zearalenone (ZEA), ochratoxin A (OTA), aflatoxin B1 (AFB1), patulin (PAT), kojic acid (KA), citrinin (CTN), T-2 toxin, deoxynivalenol (DON), ustiloxin A and ZEA metabolites α -ZOL and β -ZOL), have been studied using the larval zebrafish model (Figure 1 and Table 1).

3.1. Zearalenone and its metabolites

The most studied mycotoxin in zebrafish is zearalenone (ZEA), which is one of the worldwide most common mycotoxin that shows estrogenic activity in the range of natural steroid estrogens present in food. As reported in the literature, it can induce the yolk precursor protein vitellogenin (VTG), affect gonad development, induce atresia of oocytes and inhibition of spermatogenesis, as well as have an impact on egg production and viability, fertilization success, and sexual differentiation (Arukwe et al., 1999; Celius et al., 2000; Keles et al., 2002).

ZEA has been studied in zebrafish either in adult stages (juvenile - short-term, reproduction stages - life-long, old stages - long-term), and in early development stages (embryos and larvae). The estrogenic activity in juvenile (short-term, 42 days) and in reproduction stages (life-long exposure, 182 days) of zebrafish has been studied *in vitro* by recombinant yeast estrogen screen (rYES), physiology, morphology, and reproduction activity (i.e., spawning frequency, fecundity, fertility and hatch) (Schwartz et al., 2010) (Table 1).

To assess the relative estrogenic activity *in vitro*, it is common to use rYES with the endpoint being activation of ER-regulated genes and compared it to the natural steroid estrogens such as 17 β -estradiol (Routledge et al., 1996). ZEA short-term exposure (42 days) for rYES demonstrated a sigmoid concentration response curve reaching an effective concentration (EC) EC₅₀ ZEA of 500 μ g/L, a reduction in spawning frequency (<40%), as well as in relative fecundity (between 74% to 17%); although in life-long ZEA exposure (182 days) no EC₅₀ values of rYES curves were reached or below the detection limits (Schwartz et al., 2010). It was also noticed that relative fecundity was recovered during a ZEA depuration limit of 21 days (Schwartz et al., 2010).

Estrogenic potency of ZEA can also be measured by qRT-PCR through VTG protein levels in larvae and adults by relative abundance of *vitellogenin-1 mRNA* (*vtg-1*). Detection of VTG revealed increases between 4.4- and 8.1-folds in short-term exposure (42 days) in zebrafish exposed to high ZEA concentrations (1000–3200 ng/L); while 1.5-folds in life-long exposure (182 days) (ZEA 1000 ng/L) (Schwartz et al., 2010). Bakos et al. (2013) were able to detect VTG values for larvae and adults at exposures of ZEA as low as 0.1 μ g/L; however, female adult wild type (WT) zebrafish exposed to ZEA from 0.5 to 10 μ g/L for 21 days did not increase the VTG expression, except at the highest ZEA concentrations assayed (Table 1) (Muthulakshmi et al. 2018a).

Due to the strong relation of VTG protein for detecting estrogenicity effects at low levels in some strains, Bakos et al. (2019) developed a transgenic zebrafish line (*Tg(vtg1:mCherry)*) natural *vitellogenin-1* promoter sequence. Embryos 5 dpf of this zebrafish line were tested through a protocol based on the VTG and an estrogenic effect was detectable after seven generations and ZEA 100 ng/L, revealing the suitability of this model for detecting such effect and also the strong estrogenic capacity of ZEA (Schwartz et al., 2010; Bakos et al., 2013 and 2019).

ZEA has been studied in fish embryo toxicity (FET) in AB strain zebrafish embryos by Muthulakshmi et al. (2018b) (4hpf during 96 hpf) and by Bakos et al. (2013) (from 5 dpf to 21 dpf). ZEA LC₅₀ value was 950 μ g/L and the induction of developmental defects was observed at doses above 550 μ g/L (Table 1 and Muthulakshmi et al., 2018b). A slightly low LC₅₀ value (893 μ g/L) was obtained in embryos/larvae exposed for longer time to ZEA at different concentrations (see Table 1) (Bakos et al., 2013). In this last study, developmental effects in heart and eyes were observed as well as morphological alterations (from 500 μ g/L and above) revealing that ZEA might interfere in some ontogenic pathways. Effects related to fertility, hatch, embryo survival and gonad morphology for embryo zebrafish (42 and 182 days) exposed to ZEA at different concentrations (100 to 3200 ng/L) have not been detected in zebrafish (Schwartz et al., 2010). When studying growth indices (i.e., condition factor and hepatosomatic index) in female adult wild type (WT) zebrafish exposed to ZEA (from 0.5 to 10 μ g/L for 21 days), there was no relevant change; although reduction in gonadosomatic index and increase of caspase 3 activity were obtained. Such effects were only detectable at high ZEA concentrations, highlighting that ZEA can adversely affect the gonad functions in female zebrafish by changing growth indices, histology of the ovary, and apoptosis (Muthulakshmi et al. 2018a).

Toxicity of ZEA has been paired with oxidative stress by inducing developmental alterations, genotoxicity, and neurotoxicity (Muthulakshmi et al. 2018b), which was reported by evaluating oxidative stress and reduction of antioxidant defense by LPO, ROS, NO and GSH, GPx, GST, SOD, CAT, LDH in AB strain zebrafish embryos 4hpf ZEA treated during 96hpf (Muthulakshmi et al. 2018b). The highest values of these enzymes and molecules were reached at the highest ZEA concentration assayed (see Table 1). In a step forward of the toxicity in zebrafish embryos, neurotoxic effects through acetyl cholinesterase (AChE) activity revealed an inhibition of this enzyme, also at the highest ZEA concentrations assayed (750 and 950 µg/L). DNA damage for genotoxicity, apoptosis, and histological changes were also detectable at high doses (Muthulakshmi et al. 2018b).

In an approach to study the implications of ZEA in the hypothalamic-pituitary-gonadal (HPG) axis, RNA extraction and qRT-PCR for genes related to this axis were explored: brain genes (ERα and CYP19A1b), liver genes (ERα and VTG), and gonad genes (LHr, StAR, 3β-HSD and CYP19a1b). Zebrafish treated with ZEA showed an upregulation of these genes (see Table 1), as well as histopathological effects on ovaries at the highest ZEA concentration assayed (oocyte atresia and oocyte membrane detachment) (Muthulakshmi et al. 2018a), concluding that ZEA seems to impact the reproductive system of zebrafish through the HPG axis.

In summary, studying ZEA mycotoxin in zebrafish larvae stages reveals noticeable effects when concentrations tested were high (>700 µg/L); specifically, for development alterations, FET, enzymes detection, gene expression and neurotoxicity. When studying the main effect associated to ZEA as a potent estrogenic by rYES and VTG measurement, effects were detected at very low concentrations (~0.5 µg/L) which, if converted to human values, are easily reachable in food consumed in normal daily basis.

3.2. Ochratoxin A

OTA is produced by some species of *Aspergillus spp.* and *Penicillium spp.* and is among the most abundant food and feed contaminants (Juan et al., 201, 2016). OTA is nephrotoxic, hepatotoxic, and carcinogenic, and increases intestinal permeability which produces macromolecule trafficking through intestinal wall, which in turn strongly impairs the immune system and alters the so-called 'gut-brain' axis. It has been classified as Group 1 by IARC.

The presence of OTA has been widely documented in contaminated foodstuff, in blood samples of healthy people and of patients with chronic nephropathy, in serum samples of pregnant women, and in umbilical cord blood serum, which indicates the capability of OTA in passing the placenta barrier (Biasucci et al., 2011; Hallen et al., 1998; Minervini et al., 2013). The exposure to such compound at detectable levels highlights the potential effect in several organs at early development stages. In this sense, zebrafish exposed to OTA at embryo/larvae stages have been studied for morphological and functional effects, as well as for up/down-regulation of developmental genes.

OTA down-regulates gene of prolactin receptor (PRLR) in rats (Marin-Kuan et al., 2006). This gene is implicated in STAT pathway to promote cell growth and differentiation during

zebrafish embryogenesis (Hou et al., 2002). Wu et al (2016) verified the effect of OTA in zebrafish embryos measuring the expression of prolactin receptor (PRLRa), a gene involved in organogenesis and osmoregulation. Genes of cell growth and differentiation during development as well as the signal for the physiological control of water and electrolyte balance were evaluated. An alteration of these genes interferes with the development and function of kidneys and skin (Wu et al 2016). All these factors contributed to the disruption of liver development mediated by OTA (Wu et al., 2018) in the following sequencing: OTA activates miR-731 expression, suppresses PRLRa, and subsequently attenuates p-STAT5 and p-AKT signaling, which implies the final adverse effects on renal development and as endpoint the proliferation and differentiation of embryos.

Hepatotoxic effects of OTA have been assayed through transcriptomics on zebrafish embryos of strains WT AB and OTA treated (Wu et al., 2018). It was revealed that genes implicated in homeostasis and blood coagulation are downregulated as well as liver markers (*f7*, *f9b*, *cp* and *vtna* and the liver-specific *micro RNA-122* hepatotoxicity marker), which may result in the alteration of embryo liver size and/or impairment of its development. On the other hand, the suppression of the expression of two factors was detected, *hhx* and *prox1*, which are important transcriptional factors during hepatoblast specification. Wu et al. concluded that the interference of expression of *hhx* and *prox1* by OTA, and subsequently in the hepatocyte proliferation, results in small size of the liver and low levels of coagulation factors and *miR-122* (Wu et al 2018). This fact was relevant because genes and pathways in hepatogenesis and liver cancer in zebrafish and humans are quite maintained, which manifests the attention that should be paid in the health risk associated with population, especially in sensible groups as pregnant woman and young children.

One of the most recent studies carried out in zebrafish has been the biotransformation of OTA through microinjection of different volumes and concentrations (Table 1 and Csenki et al., 2019) in precision. OTA toxicity effects and detoxification vary according the volume injected in the yolk (from 0.22 to 4.17 nL) and OTA doses (1, 7 and 10 mg/L), and effects can be detected even after three days of exposure (Csenki et al., 2019). Mortality results were diverse although the highest mortality was observed when any volume of OTA 10 mg/L was injected. On the other hand, when observing morphological effects, all treatment at all injected volumes increased the frequency and severity of developmental deformities (Csenki et al., 2019).

FET assay for OTA has been tested at several concentrations and damage in embryos increases with the concentration of OTA (Tschirren et al., 2018). Although doses assayed by Tschirren et al. (2018) do not reflect a real scenario of the concentrations present in environment, it raises concern about the effects of OTA. Impairment was showed for OTA EC₅₀ of 0.29 and 2.65mg/L for 96- and 24-hpf, respectively; for heart rate 1.26 mg/L and for oxidative stress 0.067 mg/L, both after 72 hpf. LC₅₀ was 0.36 and 24.22 mg/L for 24 and 96hpf, respectively (Tschirren et al., 2018).

Teratogenicity of OTA and its hydrolyzed compound OTAA with zebrafish embryotoxicity assay (ZETA) was carried out by measuring different scorable endpoints (e.g., morphological deformities, hatching, behavioral responses) on zebrafish embryos. ZETA

revealed that such effect was concentration- and time-dependent even at nanomolar concentrations for OTA (Haq et al., 2016). However, for OTA α , such effect was not detectable at any concentration tested, which highlights the importance in decreasing the toxicity of OTA when it is metabolized to a hydrolyzed form (Haq et al., 2016).

In summary, the majority of the studies carried out with OTA in zebrafish larvae stages deal with the expression of genes related to renal development, proliferation of embryos, homeostasis and blood coagulation, revealing a down-regulation for most of the genes studied. Among that, deformities in development and mortality by microinjections in the sac yolk to analyze biodetoxification were reported, indicating that metabolization decreases OTA's toxicity. Embriotoxicity assays (FET or ZETA) showed higher LC50 values for OTA (0.26 mg/L and 24.22 mg/L, at different larvae zebrafish stages, Table 1) than those obtained with ZEA mycotoxin (Table 1).

3.3. Patulin

Patulin (PAT) is a mycotoxin mostly found in ripe apples and in its derivate products: fruit juices, concentrated fruit juices, reconstituted and fruit nectars, cider, apple compote and apple puree. It has also been found in other fruits although at much lower concentrations as in pears, grapes, and oranges. Commission regulation has legislated PAT at levels between 25 and 50 $\mu\text{g/kg}$, whereas for baby food products it has been set in 10 $\mu\text{g/kg}$ (Commission Regulation (EC) No 1881/2006). PAT has toxic effects due to its electrophilic reactivity which forms covalent adducts and degrading rapidly detoxification molecules as glutathione either *in vivo* or *in vitro*. It also has the capacity of forming complexes with cysteine, lysine, and histidine.

To date, there is only one study where PAT has been studied in zebrafish during 7dpf. PAT effects studied in zebrafish embryos had been antioxidant enzyme activity of CAT, GPx, and lipid peroxidation (LPO through MDA) (Table 1) (Ciornea et al., 2019). CAT and GPx activity decreased in zebrafish embryos treated with PAT, whereas LPO levels increased in a concentration-dependent manner, which indicates that there is an accumulation of ROS in concentrations that cannot be dissolved by common protective cellular mechanisms and that there is an intent of zebrafish to increase the production of antioxidant enzymes to alleviate the excess of ROS generated. Effects of PAT at neurological level have not been reported yet but some behavioral endpoints (e.g., representative locomotion, entries in the upper zone and immobility time) manifest that PAT slows the improvement of memory and enhances anxiety-like behavior (Ciornea et al., 2019).

Although the effect of PAT in zebrafish are scarce, alteration in antioxidant response as well as in the behavioral endpoint is reported in the literature. Increase in the production of antioxidant enzymes and decrease in memory and enhancement anxiety-like behavior as neurological parameters were the consequences reported in larvae zebrafish.

3.4. Citrinin

CTN is a mycotoxin known as organotoxic to kidneys, liver, and digestive apparatus. (Kumar et al., 2007; Singh et al., 2007a). The implications of CTN at developmental toxicity have been known for more than 25 years and include teratogenicity and embryotoxicity in

some animal *spp.* (e.g., rodents and dogs) with fatal final endpoints of renal malformations (Reddy et al., 1982; (Singh et al., 2007b). Literature has also reported that CTN can influence developing hearts and cardiotoxicity (Stainier and Fishman, 1994; Singh et al., 2007b). In this last direction, the effect on zebrafish was studied by Wu et al. (2013) to elucidate the mechanism of CTN cardiotoxicity in developing embryos. Embryos treated with CTN suggested alteration in the heart morphology with malformation, pericardial edema, blood accumulation, incorrect heart looping, and reduced size of the heart chambers. Heart rate and blood flow were decreased through the dysregulation of *miR-138*, retinoic acid signaling, and *tbx2a* (Wu et al 2013).

In summary, CTN exerts cardiotoxicity effects in zebrafish larvae stages have been manifested as evidenced by alteration of different heart parameters (pericardial edema, heart looping, rate,...) and in gene expression.

3.5. Aflatoxin B1

AFB1 belongs to the family of aflatoxins and is one of the mycotoxins highly present in fish feed worldwide. Its detection in aquafeed as well as the presence of its fungus producer, *Aspergillus flavus* and *A. parasiticus*, has been reported in samples from South America to Asia. Moreover, toxicological assays of potentiality for studying synergistic, addition or antagonism effects have suggested AFB1 as one of the most studied aflatoxin. Several multi-mycotoxin studies in food and feed have indicated the presence of AFB1 around 70% of the samples (Barbosa et al., 2013, Gonçalves et al., 2018). It usually appears jointly with deoxynivalenol (DON) and ZEA. On the other side, little is known from their toxicity when combined.

Few studies in zebrafish have associated AFB1 with morphological deformities and/or system alterations, e.g., loss of equilibrium, growth retardation, immunosuppression, mortality, unusual swimming patterns (Wu et al., 2019; Zhou et al., 2017; Pietsch et al., 2013). The most recent study of AFB1 in zebrafish has been carried out by Wu et al. (2019), who focused on behavior and neurodevelopment in early embryonic stage.

Viability in WT AB strain zebrafish embryos was altered after AFB1 150 ng/ mL in 7 dpf; however, for the same dose, no mortality was detected at 4 dpf (Wu et al., 2019). Swimming pattern of 7 dpf zebrafish was altered in embryos that were treated from 6 hpf with AFB1 from 15 to 75 ng/mL (Wu et al., 2019). The mobility of zebrafish was activated and slowed down at 30–75 ng/mL and 15 ng/mL, respectively.

Neurodevelopment study was carried out in 24 hpf embryos of a transgenic zebrafish *Tg (HuC:eGFP)* with AFB1. It was observed aberrant morphology of trigeminal ganglion and hindbrain neurons, alterations in levels of neurotoxic markers (*gfap* and *huC*), and in genes related to neuroactivity and neurogenesis (down-regulation of *ngfa* and *atp1b1b* and upregulation of *prtga*) (Wu et al., 2019). In consequence, it was suggested that disruption of neural formation and synapse dysfunction may be responsible for the behavioral alteration (Wu et al., 2019).

Zhou et al. (2017) studied the mortality and liver morphological effects of combined mycotoxins ZEA, DON and AFB1 in zebrafish and compared the effect of fish cells (BF-2 cells) and larvae zebrafish (WT and transgenic). Order of mortality when mycotoxins were assayed individually in BF-2 cells was, AFB1>DON>ZEA; and when assayed in zebrafish, AFB1 > ZEA > DON (Zhou et al., 2017). Morphological effects on liver were observed in the same order as for zebrafish. In binary combinations, synergistic effects were observed for AFB1 + DON and AFB1 +ZEA and antagonistic for DON + ZEA; while in tertiary combination, a synergistic effect at very low concentrations to end up with antagonism effect (Zhou et al., 2017).

Alterations reported for AFB1 in zebrafish larvae stages were related to morphology, neurodevelopment behave, swimming and neuroactivity at different ages. One of the most relevant effects reported was its synergism in liver morphology alteration when combined with DON and ZEA.

3.6. Multi-mycotoxins

The use of high-throughput screening methods for studying several mycotoxins in zebrafish might be good, although it has not been carried out yet. Zebrafish is a suitable animal model for screening mycotoxins, as mentioned in the introduction section of this review, due to its characteristics. A high-throughput chemical screen with zebrafish has been design to quantify the dynamics of food intake in live animals (Jordi et al., 2018; Sanden et al., 2012). This initial screening phase allowed to capture the complexity of whole organisms and makes understandable bioactivity and toxicity (Jordi et al., 2018), such as developmental and neurobehavioral toxicity (Krezi et al., 2018).

Screening of five mycotoxins (Ochratoxin A (OTA), T-2 toxin, deoxynivalenol (DON), and zearalenone (ZEA) and its metabolites alpha-zearalenol (α -ZEA) and betazearalenol (β -ZEA)) in adult AB strain zebrafish was carried out by Krezi et al., (2018). LC₅₀, EC₅₀, and IC₅₀ parameters were calculated and the order of toxicity was T2 > OTA > ZEA > α -ZEA > β -ZEA. (Krezi et al., 2018). Regarding developmental analysis, OTA was the only mycotoxin that did not interfere in the hatching process (until 96 hpf). Behavioral effects were only observed following exposure to OTA and ZEA and its metabolites α -ZEA and β -ZEA when exposed to a series of non-lethal doses, which demonstrated that mycotoxins are teratogenic and can influence behavior in a vertebrate model.

4. Conclusion

This review highlights the toxic effects of mycotoxin in the early developmental stages of zebrafish and highlights the effects that can be reflected in humans due to similarities of zebrafish with humans. These studies suppose an addition in the puzzle in the complex and yet incomplete mechanism of action of mycotoxins and its effects in neuronal system level. It is revealed that through the studies summarized here, there is a need in mycotoxin research to clarify potential toxic effects at neurological, teratogenic, and or degenerative levels and to increase the knowledge of the role of mycotoxins in development and function of zebrafish. Although some biological interactions of all organs and systems remain to be addressed with zebrafish, it offers wide options for the use of a complex organism instead of

in vitro techniques and replaces the use of animal models, implementing the 3Rs, as well as facilitating the assessment of mycotoxins, its translation to humans, and reducing the risks associated in clinical practice.

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Highlights:

- Zebrafish and its developing forms is a good 3Rs tool for studying mycotoxins' effects.
- Zebrafish is a vertebrate model for studying developmental and teratogenic mycotoxins effects.
- Mycotoxins effects at neuronal, teratogenic, and degenerative level are studied in zebrafish.
- Zearalenone and Ochratoxin A were the most commonly investigated mycotoxins in zebrafish.

Figure 1.-
Chemical structures of mycotoxins studied *in vitro* by using zebrafish model

Table 1. Experimental studies carried out over the last years of mycotoxins in zebrafish during early development stages.

Mycotoxin	Biological substrate	Dose (time, age)	Assay	Effect	Reference
ZEA	Zebrafish 10 months old (2 female and 4 male)	100, 320, 1000 and 3200 ng/L 42 days	a) Estrogenic potential (rYES) on days 4, 7, 12 and 21.	a) EJC50: 500 µg/L ZEA was detected between 57 and 84% and EEQ between 92 and 247%	Schwartz et al., 2010
			b) Mortality, weight, body length and gonad morphology	b) No effect on body length, survival and weight during 21 days exposed to 3200 ng/L. No effect in gonad morphology. None of the testes contained oocytes. The ovaries showed oocytes at the various stages of development without significant differences.	
			c) Reproductive performance	c) Spawning frequency: concentration dependent decrease of 98% Fecundity reduction of 116% Clutch size: negative concentration related response except for 1000 ng/L that reached the same value as control (109%)	
			d) VTG induction	d) Increase after 21 days of exposure between 4.4 and 8.1 fold at the highest concentrations tested (1000ng/L and 3200 ng/L).	
	Zebrafish (juvenile and adults)	100, 320 and 1000 ng/ L 80–440 eggs 182-day cycle Juvenile assay: Flow rate of 1L/h 140dpf Adult-reproduction assay: flow of 1920 µL/h 2 female and 4 male 84dpf	a) Estrogenic potential (rYES) on days 10, 30, 50, 70, 90, 110, and 130.	a) Juvenile: ZEA: between 46 and 86%; EEQ: 91 to 206% rYES < 80ng/L Reproduction: ZEA: 54 and 96% rYES between 78 and 109 %	Schwartz et al., 2011
			b) Viability, weight and body length	b) Juvenile For all concentrations it ranged from 62 to 80% and specially during the first month. Fish of 140dpf are not affected by ZEA 1000 ng/L revealing that ZEA exhibits a low acute toxicity in many animal species. Increase of body length, weight in females exposed to 1000 ng/L. No effect in males.	
			c) Sex ratio	Reproduction: No mortality recorded, neither in body length, weight. No effect in gonads morphology	
			d) Depuration: 21 days	Juvenile: Higher proportion of female especially after ZEA 320 and 1000 ng/L. Male/female ratio were reduced in 0.37 and 0.41, however no gonadal effect was observed Reproduction: No differences in sex ratio at any concentration assayed	
	Wild type (WT) zebrafish TL 8 adult female	0–10 µg/L 21 days	a) Somatic indices: body weight, total length, gonadal and hepatic changes	d) Increase of spawning frequency and relative fecundity denoting a capacity of recovery of reproductive output. VTG detection: increase in plasma females. In males there was a complete depuration after 21 days. a) Gonadal change only at high doses (5 and 10 µL). Ovary morphology changes with degenerated follicles	Muthulakshmi et al., 2018a

Mycotoxin	Biological substrate	Dose (time, age)	Assay	Effect	Reference
	AB strain zebrafish 50 embryos 4hpf	350–950 µg/L	b) Apoptosis (Caspase-3, in ovaries) c) RNA extraction and quantitative rt-PCR for genes of hypothalamic-pituitary-gonadal axis d) Histological examination a) Development toxicity (24, 48, 72 and 96 hpf) and heart rate measurement (48, 72 and 96 hpf) 10 embryos per group	b) At high doses caspase-3 activity increased significantly. This suggested that ZEA could activate caspase-3 to induce apoptosis. c) Brain genes (ERα and CYP19A1b), liver genes (ERα and VTG) and gonad genes (LHR, SiAR, 3P-HSD and CYP19A1b) increased their expression at ZEA 5 and 10 µg/L. d) At a higher concentration (10µL) there was irregularity in membrane attachment and oocyte atresia. a) At 24 and 48hpf no deformation was observed below 550 µg/L; while at higher hpf spine curvature was noted. Pericardial edema, hyperemia, yolk sac edema and spine curvature at 750 and 950 µg/L at 72 and 96 hpf. Abnormal swimming behavior in circular movement in the embryos of 750 and 950 µg/L ZEA After 48hpf a marginal increase was observed at 950 µg/L, while no effect was observed at 72 hpf. Decrease of heart rate in embryos at 96 hpf for ZEA 950 µg/L	Muthulakshmi et al., 2018b
			b) Biochemical and antioxidant markers (96 hpf): ROS (10 embryos), LPO (20 embryos), NO, GSH (20 embryos), SOD, CAT, GPx, GST (8 embryos), LDH (8 embryos), AP and protein. c) Neurotoxicity (96 hpf): AChE activity (72 hpf), Comet assay, apoptosis and histopathology.	b) -Increase of i) ROS and LPO at 750 and 950 µg/L; ii) NO and LDH at 950 µg/L. -Reduction of SOD, CAT, GPx, GST and GSH at ZEA highest concentrations assayed (750 and 950 µg/L) -AP activity greatly decreased at the 950 µg/L.	
				c) Inhibitory effect on AChE activity at the highest concentrations assayed (750 and 950 µg/L). -Comet assay: DNA damage in zebrafish embryos exposed to 750 and 950 µg/L. -Apoptotic cells at 950 µg/L. -Histology: Embryos shown various changes like morphological alteration, yolk sac edema, swim bladder inflation and abnormal muscle fibers and body curvature in the highest concentration (950 µg/L).	
	Zebrafish: - Embryos 1hpf - Mature male adults	Embryos: 1 ng/L to 5000 µg/L From 24 to 120hpf Adults (n=7): 0.1 to 1000 µg/L 21 dpf	a) Embryotoxicity tests. Survival and lethal endpoints (coagulation, missing heartbeat, tail detachment and developmental abnormalities) LC₁₀ and LC₅₀ (24 well/plate, 1 embryo/well)	a) 24hpf: for ZEA 1500 µg/L embryos showed pericardial edema and at 5000 µg/L all embryos stopped development at the tail bud stage after 10h of exposure. 48hpf: for ZEA 500 µg/L embryos showed dorsal curvature of the body axis and abnormal heart and eye development (estrogenic effects). Yolk sac and pericardial edema from 1000 µg/L. 72hpf: embryos hatched for ZEA 1000 µg/L. Edema was detected at 100 µg/L. From 500 µg/L and above pigmentation was reduced, altered eye and heart development and dorsal curvature of the body axis. Degree of curvature was dose dependent (EC₅₀= 596 µg/L 96 to 120 hpf: edema from 50 µg/L, curved body axis from 500 µg/L and reduced pigmentation from 50 µg/L. LC₁₀: from 335 to 1424 µg/L LC₅₀: from 893 to 2854 µg/L	Bakos et al., 2013
			b) RNA isolation (10 larvae or 1 whole body adult) for rt-PCR: β-actin (control gen) and vitellogenin	b) All ZEA concentrations induce VTG-1 mRNA expression in a time-dependent manner (120hpf). Highest value was measured at 250 µg/L (median effective concentration 3.24 µg/L Low level of VTG was also detected in the negative control.	

Mycotoxin	Biological substrate	Dose (time, age)	Assay	Effect	Reference
OTA	Zebrafish estrogen sensitive liver transgenic line Tg(VTG:mCherry) From 3 to 5 dpf	From 0.1 to 500 µg/L (8 conc.) 20 embryos/conc.	c) ELISA (in adults) (vtg protein levels) a) Testing mCherry inducibility of fluorescent b) Dose-response (sensitivity)	c) All ZEA concentrations induce VTG mRNA Comparison of results revealed that ELISA and rt-PCR methods correlate well as well larval and adult VTG induction rates. a) There was an inhomogeneity and a large variation of response b) Concentration dependent fluorescent response. LOEC at 0.1 µg/L Relative sensitivity: 0.78 EC10, EC50 and EC90 were 3.18, 10.75 and 35.17 µg/L, respectively.	Bakos et al., 2019
	Zebrafish embryos of WT, AB strain and Tg(wt1b:GFP)	0.1–1 µM 96 well/plate: 1 embryo/well 24 well/plate: 10 embryos per well 6hpf	a) Survival rate (LD50) (96 well/plate) b) Whole-mount immunostaining for measuring the heart chambers (with MF20 and S46) (24well/plate) 6 to 72 hpf c) Renal function: Dextran clearance assay (microinjection) (images at 0 and 7hpf) (96 well/plate, survival embryos after 72hpf OTA treatment) 0.1 to 0.5 µM d) RNA sequencing (RNA- seq) 6 to 48hpf 0.5 µM e) qRT-PCR for microRNA and antagomiR-731 microinjection (performed into the yolk- stream of 1–2 cell stage embryos). 25 embryos per dose 6hpf a) Assessment of the viability of embryonic zebrafish (6hpf) b) mRNA for genes involved in hemostasis and blood coagulation:	a) LC50: i) from 6 to 72hpf: 1 µM; ii) from: 24 to 96hpf: 0.97 µM. OTA at different time points lead to a slight difference in embryo's viability. Dose higher than 0.5 µM OTA exhibited pericardial edema, dark yolk edema and yolk sac edema. b) It was also shown that OTA caused defects in heart chambers at 72hpf: i) 0.25 µM separates and stretches out ventricle and atrium and ii) 1 µM increases the distance between sinus venosus and bulbus arteriosus 1.5-folds. c) Pronephros had shrunk and arranged in a disorganized manner at 0.5 µM OTA reduce the glomerular filtration in embryonic status from 55% to 31% for 0.25 µM OTA and 11 % for 0.5 µM OTA. d) OTA inhibited the expression of PRLRa in a dose- dependent manner (0.25 µM OTA to 47% after 24hpf). OTA reduce the phosphorylated levels of STAT5 in a concentration- dependent manner: i) 53% for 0.25 µM and ii) 38% for 0.5 µM. OTA suppressed the phosphorylation of the AKT protein and reduced the levels of serpinal levels. OTA modulates the PRLRa/STAT5/serpinal pathway in the embryonic zebrafish. Embryos 0.5 µM OTA-treated for 42hpf caused up-regulation of 7 microRNAs and down-regulation of 5 microRNAs. e) -OTA induced miR-731 expression in a dose-dependent manner at 48hpf (higher peaks). -The antagomiR-731 recovered the levels of PRLRa reduced by OTA (from 17 to 26%) and enhanced the PRLRa mRNA levels to 147% in the absence of OTA. PRLRa is a target gene for miR-731 a) The viability decreased when increasing the exposure time and the OTA concentrations to 87% (0.05 µM OTA) and 58% (0.1 µM OTA) 24hpf. OTA > 0.25 µM had pericardial edema and dark yolk sac at 96 hpf. b) Genes which participate in coagulation cascade (fga, fgb, fgf, f2, f7, f7i, f9b, f10, fig and proc) where decreased upon OTA treatment.	Wu et al., 2016

Mycotoxin	Biological substrate	Dose (time, age)	Assay	Effect	Reference
			<i>fga, fgb, fgg, f2, f7, f7i, f9b, f10, flg</i> and <i>ef</i>. From 6hpf to 48hpf 0.5 µM c) rt-PCR for <i>f7, f7i, f9b, cp, vtna</i> and <i>mir-122</i> From 6 to 72hpf d) Liver fluorescence imaging e) Liver tissue observation and histology (96 hpf and 8dpf) f) Whole-mount <i>in situ</i> hybridization g) Phospho-histone H3 immunohistostaining a) Assessment of development: mortality, development stage, eyes, somites, tail movement and occurrence of oedema (72hpf) Pigmentation and blood flow, heart beats (20–30 sec) at 48 and 72hpf b) Measurement of ROS (72hpf) a) Mortality: coagulation, lack of somite formation and lack of heart function b) Sublethal: pericardial edema, yolk edema, tail deformation, craniofacial deformation and disintegrated abnormal embryo shape	c) OTA decreased the expression of liver specific genes: <i>f7, f7i, f9b, cp, vtna</i> and the expression of <i>mir-122</i>. OTA toxicity may result in impairment of liver development in embryos. d) OTA (0.1 and 0.5 µM) reduced fluorescence in the 96 and 72 hpf embryos. e) 0.25 µM OTA: Caused variously- sized clear vacuoles. 0.5 µM OTA: Cytoplasmic cavitation, nuclei were located at the margins of hepatocytes and the ratio of nucleus to cytoplasm was increased. f) OTA (0.25 and 0.5 µM) reduced with the <i>hhex</i> and <i>prox1</i> expression and it altered hepatocyte proliferation. g) The number and the percentage of phosphorylated histone H3 positive cells on liver sections was reduced in the OTA group at 96 hpf. a) OTA induced dose-dependent mortality in embryos. EC₅₀ went from 0.29 to 2.65 mg/L for 96hpf and 24hpf, respectively. LC₅₀ went from 0.36 to 24.22 mg/L for 24 and after 96hpf. The hatching was happening at 72 and 96hpf and exposures from 0.16 to 0.63 mg/L; however higher OTA doses did not succeed at OTA 1.25 mg/L or higher. It was observed a correlation between OTA concentrations and the decrease of the heart rates. After 48 and 72h of exposure from 0.63 mg/L at 72h no heart rates were detected after 2.5 mg/L b) OTA increased oxidative stress in treatments higher than 0.078 mg/L. a) Mortality increased along with the injection volume for all three OTA concentrations at 72hpf; no change at 120 hpf. Mortality was i) maximum for 1 mg/L when 4.17 nL were injected, ii) increased progressively at 7mg/L with different volumes and when 4.17nL were injected caused the higher mortality and ii) maximum mortality with low volumes of 10 mg/L. OTAa: increased mortality with the injected volume. At 120hpf a dose-response relationship was found between the injected volume and embryo mortality. Mortality increased with the injection with the highest volume (38.5%). b) All treatments with all injected volumes increased the frequency and severity of developmental deformities. At 72 hpf: the highest deformity was obtained at 7 mg/L OTA and highest volume injected. At 120 hpf: deformed embryos was the highest in those OTA treated from 0.52 nL and above. 72hpf 1.77 nL 7 mg/L OTA embryos displayed craniofacial deformities, small eyes, curvature of the body axis, yolk deformities, reduced growth rates and edemas. 120hpf: symptoms and deformation got more severe and appear in all body	Tschirren et al., 2018 Csenki et al., 2019

Mycotoxin	Biological substrate	Dose (time, age)	Assay	Effect	Reference
OTA OTA α	Zebrafish embryos	OTA: 0.05–2.5 μ M OTA α : 0.1–2.5 μ M 24well/plate 5 embryos/well (<2hpf, 4–32 cell stage)	a) Zebrafish embryo toxicity assay: ZETA (2–5 dpf)	a) OTA at 1 μ M developed abnormally embryos by 1dpf which increased at subsequent time-points. All embryos were deformed at OTA concentrations of 1 (2dpf), 0.25 μ M (4dpf) and 0.1 μ M (5 dpf) Teratogenicity was observed at sub-micromolar concentrations (0.05 μ M by 5dpf) EC ₅₀ for teratogenicity of OTA decreased from 1 to 5 dpf from 0.25 to 0.02 μ M, respectively. OTA higher than 0.5 μ M was lethal by 2dpf and surviving embryos were severely retarded. OTA 0.05 μ M and 4–5 dpf morphological deformities were observed characterized by reduced growth rates, craniofacial deformities, curvature of the body axis and edemas as well as dose-dependent reduction in hatching rates. OTA has acute toxicity but OTA α had no significant effect in embryo development as no mortality of embryos were detected after 5dpf.	Haq et al., 2016
PAT	Zebrafish	PAT: 70 μ g/L 7 days	a) CAT b) GPx c) LPO (MDA measurement). d) Behavioral parameters (representative locomotion, entries in the upper zone and immobility time)	a) PAT decreased the CAT activity to 7.2 mg protein (100mg/L), respectively. b) PAT decreased GPx activity respect the control (0.17 U/mg) at 100 mg/L with values of 0.11 U/mg. c) MDA concentration was 0.97 nmol/mg. d) PAT attenuate the swimming all over the tank in a dose-dependent manner; decreased the number of entries in the upper zone and increase the immobility time Locomotor activity: PAT increased this locomotion Such effect reflected an increase of spontaneous alterations percentage for PAT. PAT stimulated the memory formation by slowly improving memory and enhance of anxiety-like behavior.	Giomea et al., 2019
CTN	Wild type AB zebrafish embryos Heart-specific transgenic line Tg(BMP4:EGFP) Erythrocyte-specific transgenic line Tg(gata1:dsRED) ^{y42}	20 and 50 μ M 24, 72 and 120 hpf	a) Viability (from 24 hpf to 120 hpf) Whole-mount immunostaining 10 AB embryos 24 to 72hpf b) Assessment of SV-BA distances in Tg(BMP4:EGFP) c) Observation histological transverse sections d) Cardiac functions of zebrafish embryos: *heart rate (48 and 72 hpf) 15 embryos	a) Viability for 50 μ M was 71.6% at 72hpf but all dead after 120 hpf; while for 20 μ M lethal effect at 72hpf while at 120 hpf viability was 38% CTN altered the phenotypes of developing heart, including abnormal tube looping, reduced chamber sizes and pericardial edema. b) The SV-BA distance decreased in embryos exposed to CTN 50 μ M. c) It was also observed the cardiac defects. d) The heart rate decreased 70% after 50 μ M CTN CTN decreased the blood flow rate of embryos. CTN altered the expression of heart-related genes (tbx2a, nkx2.5, bmp4 and jun4).	Wu et al., 2013

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Mycotoxin	Biological substrate	Dose (time, age)	Assay	Effect	Reference
AFB1 DON ZEA AFB1 + DON AFB1 + ZEA DON + ZEA AFB1 + DON + ZEA	WT and Tg(Ulfp10ads Red; elafA:egfp) (LiPan) zebrafish larvae 48hpf *transgenic was developed to study embryonic development and in vivo screening for hepatotoxins	Individual: AFB1: 0–1.28 µM DON: 0–135 µM ZEA: 0–18.85 µM Mixtures: AFB1: 0.04 µM DON: 67.5 µM ZEA: 6.28 µM	a) Viability (LD₅₀) from 48 to 120 hpf 96 well/plate, 1 embryo/well b) High content screening for multi-parametric cytotoxicity assessment. c) Imaging-morphology (Analysis by HCS)	In general, β-ZOL affected the swimming parameters in all tested ages and 100hpf was the age that increased the distance moved, time spent active and decrease of mean swimming speed. T2 and DON did not affect larval behaviour or locomotor activity. a) <u>Individual</u>: AFB1 was the most toxic mycotoxin. AFB1 and ZEA increased toxicity as the exposure time progressed. AFB1 induced mortality on 72hpf 1.28 and at 96hpf 0.64 µM. ZEA induced mortality from 48 to 120 hpf at 9.43 µM and increase along the exposure period. NOAEL at 120hpf: AFB1 0.16 µM; ZEA 6.28 µM. Mixtures: AFB1 + DON at 96 and 120hpf reduced embryo viability. AFB1 + ZEA at 96hpf reduced embryo viability and at 120 hpf killed all embryos. DON + ZEA: no mortality observed AFB1 + DON + ZEA: slightly decreased the viability during 96hpf and decreased even more at 120hpf. b) <u>Individual</u>: Liver size: decrease at ZEA 12.6 µM (73.5%). AFB1 and DON did not produce any significant change in liver area. RFP intensity: i) decreased dose-dependently at 0.04 µM AFB1 (66%); ii) decreased at 33.75 and 67.5 µM DON and iii) ZEA at 6.28 and 12.57 µM 31 and 41% respectively. Mixtures: Decreases in liver areas for AFB1 + DON, AFB1 + ZEA and AFB1 + DON + ZEA from 63 to 54%. DON + ZEA increased liver area by 51% RFP intensity: decreased for AFB1 + DON, AFB1 + ZEA and AFB1 + DON + ZEA from 62 to 56%. c) <u>Individual</u> treatment revealed moderate edema around the area of yolk sac and slightly upward bending for all mycotoxins. Mixtures: AFB1 + DON and AFB1 + ZEA strong bending of body trunk, yolk sac edema and reduced pigmentation. Pericardial edema for AFB1 + ZEA. AFB1 + DON + ZEA slight bending of body trunk, yolk sac edema and reduced pigmentation. DON + ZEA: slight yolk sac edema and bending of tail tip.	Zhou et al., 2017

AChe: Acetylcholinesterase.

AFB1: Aflatoxin B1

AP: Acid phosphatase

CAT: Catalase

CIT: Citrinin

DON: Deoxynivalenol

EC50: concentration that caused any deformity in 50% of larvae

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EEQ: estrogen equivalent

ELISA: enzyme-linked immunosorbent assay

Fga: Fibrinogen alpha chain

Fgb: Fibrinogen beta chain

Fgg: Fibrinogen gamma chain

Plg: Plasminogen

GLU: Glucose

GPx: Glutathione peroxidase

GSH: Reduced glutathione

GST: Glutathione S-transferase enzyme

HCS: High content screening

ID50: concentration interfering with hatching in 50% of larvae

LC50: concentration that causes lethality in 50% of larvae

LDH: Lactate dehydrogenase

LHr: Luteinizing hormone receptor

LOEC: Lowest effective concentration

LPO: Lipid peroxidation

MTT assay: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide assay

NO: Nitric oxide

OTA: Ochratoxin A

PAT: Patulin

PCR: Polymerase Chain Reaction

Proc: Protein C

qRT-PCR: Quantitative real time PCR

ROS: Reactive oxygen species

rt-PCR: Real time PCR.

rYES: Recombinant yeast estrogenic screen

StAR: Steroidogenic acute regulatory protein

T-2: Trichothecene mycotoxin

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TBARS: Thiobarbituric acid reactive substances
Tg(LFABP:EGFP); fluorescent hepatocytes
VTG-1: Vitellogenin 1 gene
ZEA: Zearalenone
ZETA: Zebrafish embryo toxicity assay
 α -ZOL: Alpha-zearalenol
 β -ZOL: Beta-zearenol
 3β -HSD: 3β -hydrosteroid dehydrogenase-1

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