Mixture Toxic Impacts and the Related Mechanism of Aflatoxin B1 and Deoxynivalenol on Embryonic Zebrafish (Danio Rerio)

Yanhua Wang
Zhejiang Academy of Agricultural Sciences
Qiang Wang
Zhejiang Academy of Agricultural Sciences
Chun Ji
Tongzhou District Comprehensive Food Inspection Center
Xiaoxuan Guo
Chinese Academy of Agricultural Sciences
Guiling Yang
Zhejiang Academy of Agricultural Sciences
Dou Wang
Zhejiang Academy of Agricultural Sciences
Hongbiao Weng
Zhejiang Academy of Agricultural Sciences
Yongzhong Qian
Chinese Academy of Agricultural Sciences
Chen Chen (chenchen02@caas.cn)
Institute of Quality Standard and Testing Technology for Agro-Products Chinese Academy of Agricultural Sciences https://orcid.org/0000-0003-2599-8332

Research

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Abstract

Background

Although humans and animals are often simultaneously exposed to a variety of mycotoxins via feed and food consumption, in which aflatoxin B1 (AFB1) and deoxynivalenol (DON) is one of the most prevalent combination of mycotoxins. Many toxicological reports have merely focused on the impacts of single mycotoxins. In the present study, the combined toxicity and the related mechanism of AFB1 and DON to zebrafish (Danio rerio) were investigated.

Results

We showed that DON had lower toxicity to embryonic zebrafish with a 7-day LC_{50} of 218.3 mg a.i. L^{-1} in comparison to AFB1 (0.031 mg a.i. L^{-1}). The mixture of AFB1 and DON elicited an additive combined effect on zebrafish embryos. The levels of CAT, caspase-3, and T4 markedly varied in most single and mixture groups. The expressions of four genes (cas3, apaf-1, cc-chem, and cyp19a) associated with oxidative stress, cellular apoptosis, immune system, and endocrine system were markedly varied upon the mixture exposure in comparison to the corresponding single exposure of AFB1 or DON.

Conclusions

Our results demonstrate that the impacts of a mixture could not be estimated solely based on toxicities of the single mycotoxins. Taken together, our comprehensive investigation on the mycotoxin mixtures and their potential mechanisms could better reflect the reality of mycotoxin contamination in food and feed.

1. Introduction

As toxic secondary metabolites generated by a wide variety of fungi, mycotoxins can contaminate food and feed resources, which has become a worldwide problem to public health and economic significance [1]. These toxicants can cause acute toxicity or chronic disorders in animals and humans, including reduced food production and livestock breeding and induction of cancers and immune deficiency [2, 3]. Most toxin-producing fungi can generate various mycotoxins simultaneously and result in the co-occurrence of multiple mycotoxins in different feed and food commodities in which aflatoxin B1 (AFB1) and deoxynivalenol (DON) is one of the most prevalent combination [4–6]. Although humans and animals can be exposed to multiple mycotoxins through the consumption of contaminated foods and feeds, the great majority of studies merely focus on the toxicology of individual mycotoxins, which underestimates the combined toxicities caused by the mixtures [7]. Therefore, the adverse effects of mixture mycotoxins are be growing concern and needs to be elucidated. However, mixture toxic effects of mycotoxins on *D. rerio* have not been well documented.

Although numerous studies have investigated the mixture effects of mycotoxins lately, most of these reports have focused on cytotoxicity testing [8]. The mixture of AFB1 and DON can decrease the viability

of cells by inducing intracellular reactive oxygen species (ROS) production and promoting apoptosis in rat liver cells, which was mediated by apoptosis pathway shared between AFB1 and DON [9, 10]. However, the cytotoxicity testing method is restricted by immortalization, limited survival, metabolic imbalance, or lack of tissue intercourse [11]. Zebrafish (*Danio rerio*) has been well accepted as general fish and vertebrate model to investigate the chemical toxicity and mechanism because of its easy breeding in the laboratory, great fecundity, fast development, short life cycle, and transparency of the eggs and embryos [12]. Therefore, it is an outstanding vertebrate model due to its highly developmental similarity to mammals [13]. Because of these reasons, toxicity evaluation on zebrafish can serve as a significant reference to humans [14].

Aflatoxins are a family of closely related pyranocoumarin compounds generated as secodary metabolites of the common molds *Aspergillus flavus*, *A. parasiticus* and to a lesser extent *A. nominus* [15]. As the most toxic xenobiotic and potent carcinogen, AFB1 has hepatocarcinogenic, mutagenic, and genotoxic effects [7]. Deoxynivalenol is a trichothecene, and it is mainly produced by Fusarium [16]. Ingestion of DON-polluted feed can cause anorexia, and vomiting, and affect the immune system in different livestock species [17]. Since AFB1 and DON frequently occur as mixtures in various food and feed commodities, which may have additional impacts on humans in comparison to their respective compounds [18]. Up to date, the mechanism underlying the mixture impacts in *D. rerio* remains largely unclear [19]. Thus, we investigated the hidden effects of these two mycotoxins on zebrafish embryos. Such systematic tests laid a solid foundation for future studies on the toxicological impacts and mechanism of mycotoxin mixtures on humans.

2. Materials And Methods

2.1. Materials

AFB1 (purity ≥ 99%, CAS Number: 1162-65-8) and DON (purity ≥ 99%, CAS Number: 51481-10-8) were purchased from Sangon Biotech (Shanghai) Co., Ltd. (China). A stock solution of AFB1 was prepared in dimethylsulfoxide (DMSO) and Tween-80, and the stock solution of DON was prepared in Milli-Q water directly. Mycotoxins of desired concentrations were prepared using reconstituted water supplemented with 2 $mmol{\cdot}L^{-1}$ Ca^{2+}, 0.5 $mmol{\cdot}L^{-1}$ Mg^{2+}, 0.75 $mmol{\cdot}L^{-1}$ Na^{+}, and 0.074 $mmol{\cdot}L^{-1}$ K^{+} [20].

The contents of malonaldehyde (MDA), lipid peroxidation (LPO) enzyme, antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD)], apoptotic enzymes (caspase-3 and caspase-9), and detoxification enzymes [cytochrome P450 (CYP450), carboxylesterase (CarE) and glutathione-S-transferase (GST)], as well as the vitellogenin (VTG) and thyroid hormones (THs), such as triiodothyronine (T3) and thyroxine (T4), were determined using ELISA kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TransZol Up Plus RNA Kit and ransScript® II One-Step RT-PCR SuperMix were purchased from TransGen Biotech (Takara, Dalian, China). All other chemicals and compounds were of analytical grade.

2.2. Maintenance and rearing conditions of fish

Wild-type (AB strain) adult zebrafish were obtained from the China Zebrafish Research Center (Wuhan, China) and reared in a laboratory-scale polyisoprene tank (200 L) equipped with a recirculation system. The fish breeding was carried out at 27 ± 1℃ under a photoperiod consisting of 14 h of light and 10 h of darkness. Fish were fed TetraMin Flake (fish food) complemented with brine shrimp (*Artemia salina*) twice a day. For egg induction, parental zebrafish (male: female ratio, 1: 2) were segregated in spawning boxes (Esen Corp, Beijing, China) overnight. Light-induced spawning was carried out the next morning, and eggs were harvested within half an hour. Fertilized and morphologically normal eggs were picked for further assays. The animal study was authenticated by the Animal Care and Use Committee of Zhejiang Academy of Agricultural Sciences (Hangzhou, China).

2.3. Acute toxicity determination

2.3.1. Individual mycotoxin toxicity determination

The acute toxicity test on embryonic fish was carried out based on OECD test guideline 236 with minor modifications [21]. The median lethal concentration (LC_{50}) was determined using the preliminarily tested concentrations. Embryos at about 3 h post-fertilization (hpf) were stochastically selected and placed into 96-well plates, and each well contained one embryo and 2 μL of exposure reagent. Reconstituted water was employed as untreated (blank untreated) control, and solvent untreated containing the same content of DMSO and Tween-80 at the highest concentration was adopted in the AFB1 test. Each test concentration and untreated control were performed three times. Each replicate was composed of 32 wells. Embryos were challenged by six different concentrations with a geometric ratio to establish the concentration-mortality relationship for each mycotoxin. All 96-well plates were incubated at the conditions same as the rearing conditions. The plates were sheathed by transparent lids for 7 days. The test solution was changed every 12 h to preserve the concentrations of toxicants. The mortality was determined according to the signs of coagulation of embryos, lack of somite formation, non-detachment of the tail, and failure of heartbeat, and recorded after 7 days of exposure.

2.3.2. Determination of mixture toxicity

The mixture toxicity of mycotoxins was examined with zebrafish embryos. In this study, an equitoxic ratio was adopted to avoid that when the toxicity of a single pollutant differed greatly, one pollutant would dominate and mask the toxic effect of the other pollutant, thus changing the combined action type of mixed pollutants. Based on the LC_{50} values acquired in the single toxicity determination, the final concentration of the individual mycotoxin in the mixture was used as the corresponding LC_{50} value (equipotent combinations) to assess the mixture toxicity and type of interaction. The total concentration of each blend was methodically altered, and all the above-mentioned ratios were unchanged to explore the concentration-response correlation. All experiments were performed three times for each concentration. The other test routines of mixture toxicity determination were consistent with those of the individual mycotoxin test.

2.4. Subacute toxicity determination

The subacute toxicity was determined based on the data of acute toxicity to embryos to explore the potential mechanisms of AFB1, DON, and thebinary mixture in embryonic zebrafish.

2.4.1. Exposure procedure

Briefly, 1/320, 1/80, and 1/20 of 7-day LC_{50} for each mycotoxin were defined as the low, middle, and high concentrations, respectively. Accordingly, low, middle, and high concentrations in the combined exposure of AFB1 + DON (JOT) were combinations of AFB1 and DON at the low, middle, and high concentrations, respectively. Besides, the blank and solvent controls were set up. About 300 embryos of 3 hpf were stochastically picked and placed into a 1-L beaker containing 600 mL mycotoxin solution, and each experiment was repeated three times for each concentration. The test solution was renewed every 12 h. After exposure for 1 week, hatched larvae were collected and rinsed twice with reconstituted water.

2.4.2. Biochemical level determination

Briefly. 200 larvae were homogenized (1:20, w/v) in 50 mmol·L^{-1} potassium phosphate buffer (pH 7.0) containing 0.5 mmol·L^{-1} EDTA, followed by centrifugation at 12,000 rpm for 30 min at 4°C. The supernatant was collected and used for the assay of biochemical factors.

The indexes of oxidative stress, including MDA content, the activities of LPO enzyme, antioxidant enzymes including SOD, CAT, and POD, apoptosis-related enzymes including caspase-3 and caspase-9, and detoxification enzymes including CYP450, CarE, and GST, as well as the levels of VTG and THs (T3 and T4), were determined according to the instructions of the respective assay kits.

2.4.3. Molecular level determination

After 7 days of exposure, 30 embryos were harvested and dissolved in Trizol reagent (Takara, Dalian, China) for total RNA isolation with a previously described protocol [22]. The RNA concentration was determined at 260 nm, and the RNA integrity was confirmed based on the absorbance ratios (OD260/OD280). Purified RNA was reversely transcribed into cDNA using a PrimeScript™ RT reagent Kit (Takara, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a CFX Real-Time PCR Detection System (Bio-Rad, USA) using a SYBR Green PCR Master Mix reagent kit (Takara, Dalian, China). After an initial denaturation step at 95°C for 30 s, amplifications were carried out with 40 cycles at a melting temperature of 95°C for 5 s, and an annealing temperature of 60°C for 30 s. The relative expressions of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. *β*-actin was adopted as the internal control. All primers were designed by Primer 6.0 software and synthesized by Sangon Biotechnology (Shanghai, China) (Supplemental Table S1).

2.5. Statistical analysis

A probit analysis was carried out to determine the acute toxicity of mycotoxins to *D. rerio* using a previously developed program [23]. The differences between the control and exposure groups were

determined using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc comparison, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Individual and mixture acute toxicity evaluations

The acute toxicity data of AFB1 and DON to embryos of *D. rerio* is listed in Table 1. We found that various mycotoxins had significantly different toxicities to fish. At a 4-day interval, AFB1 exerted higher toxicity to *D. rerio* than DON. At a 7-day interval, AFB1 still elicited higher toxicity to zebrafish than DON. The toxicity of AFB1 was 7,042 times higher in comparison to DON after exposure for 7 days. Moreover, the toxicities of AFB1 and DON were significantly elevated when the exposure period was extended. To understand the interaction between different mycotoxins in the mixture toxicity against *D. rerio*, we detected the LC_{50} values of AFB1 and DON in their mixture after exposure for 7 days. According to the LC_{50} values of AFB1 and DON individually and in the mixture, the mixture toxicity of AFB1 and DON displayed an additive effect on *D. rerio*.

Table 1
Individual and combined toxicities of aflatoxin B1 and deoxynivalenol to embryonic fish.

Exposure time (d)	LC_{50} (95% FL)[a] mg a.i. L^{-1}		LC_{50} (95% FL)[b] mg a.i. L^{-1}		AI[c] value
	aflatoxin B1	deoxynivalenol	aflatoxin B1	deoxynivalenol	
4	0.087(0.065 ~ 0.12)	> 1000	-[d]	-	-
7	0.031(0.017 ~ 0.042)	218(164 ~ 301)	0.018(0.013 ~ 0.021)	126(91 ~ 147)	-0.16

[a] The LC_{50} (95% fiducial limit) for aflatoxin B1 or deoxynivalenol individually.

[b] The LC_{50} (95% fiducial limit) for aflatoxin B1 or deoxynivalenol in the mixture.

[c] AI additive index.

[d] – no determined.

3.2. Biochemical level determination

3.2.1. Oxidative stress determination

The MDA level in the high-concentration group of AFB1 and the low-concentration group of DON was surprisingly inhibited in comparison to the control group. Besides, a markedly lower level of MDA was also detected in the middle-concentration group of JOT in comparison to the corresponding single

exposure groups of AFB1 and DON (Fig. 1A). The LPO activity was radically increased in the high-concentration group of AFB1. By contrast, the activity was markedly elevated in the low-concentration group of DON. Moreover, its activity was also radically decreased in the middle-concentration group of JOT in comparison to the corresponding single exposure group of DON (Fig. 1B). The SOD activity was not outstandingly altered in all the single and JOT exposure groups. Additionally, no outstanding alteration was monitored in all the JOT exposure groups in comparison to the corresponding single exposure groups of AFB1 or DON (Fig. 1C). The CAT activity remained unchanged in all the single exposure groups. However, the activity was markedly decreased in the high-concentration group of JOT in comparison to the corresponding single exposure group of DON (Fig. 1D). The POD activity was diminished in the low-concentration group of DON in comparison to the control group. On the contrary, the activity was remarkably increased in the low-concentration group of JOT in comparison to the corresponding single DON exposure group (Fig. 1E).

3.2.2. Apoptosis and detoxification enzyme activities

The activity of caspase-3 was prominently induced in the middle-concentration group of AFB1 in comparison to the control group. Besides, its activity was prominently induced in the low-concentration group of JOT in comparison to the corresponding single exposure group of DON (Fig. 2A). Similarly, the caspase-9 activity was not noteworthily varied in all the single and JOT exposure groups. Besides, its activity was not markedly changed in all the JOT exposure groups in comparison to the corresponding single exposure groups of AFB1 or DON (Fig. 2B). The CYP450 activity was conspicuously reduced in the high-concentration group of AFB1. In contrast, its activity was conspicuously increased in the high-concentration group of JOT in comparison to the corresponding single AFB1 group (Fig. 2C). Similar to the CAT activity, the CarE activity was not observably different in all the single exposure groups in comparison to the control group. However, the level was observably elevated in the high-concentration JOT group in comparison to the corresponding single DON group (Fig. 2D). The GST activity was extraordinarily enhanced in the low-concentration groups of AFB1 and JOT, and the high-concentration group of DON. By contrast, its activity was extraordinarily inhibited in the high-concentration JOT group in comparison to the corresponding single DON group (Fig. 2E).

3.2.3. TH and VTG levels

The T3 level was manifestly reduced in the low-concentration group of DON in comparison to the control group and the corresponding JOT exposure group. Moreover, its level was dramatically decreased in the high-concentration group of AFB1 in comparison to the control group and the corresponding JOT exposure group. Contrarily, its level was manifestly elevated in the high-concentration group of DON in comparison to the control group (Fig. 3A). The T4 level was enormously lessened in the low-concentration group of DON and the high-concentration group of AFB1 in comparison to the control group. Nonetheless, its level was enormously increased in all the JOT exposure groups in comparison to the control group. Its level was also enormously augmented in the middle- and high-concentration groups of JOT in comparison to the corresponding single exposure groups of AFB1 (Fig. 3B). The VTG level was

drastically decreased in the middle-concentration groups of AFB and DON in comparison to the control groups. Besides, its level was remarkably reduced in the low-concentration group of DON in comparison to the control group. Conversely, its level was drastically elevated in the high-concentration group of JOT in comparison to the control group (Fig. 3C).

3.3. Determinations of gene expression

3.3.1. Influence on genes involved in anti-oxidative stress and cell apoptosis

The expression level of *Mn-sod* was markedly increased in the middle-concentration groups of DON and JOT, as well as in the high-concentration groups of AFB1 and DON. However, its expression was sharply decreased in the high-concentration group of JOT in comparison to the corresponding single AFB1 group (Fig. 4A). The expression of *Cu/Zn-sod* was remarkably elevated in the middle-concentration groups of AFB1 and DON in comparison to the control group. Additionally, the level of expression was remarkably induced in the high-concentration group of AFB1 (Fig. 4B). The *gpx* expression was substantially elevated in the low-concentration group of DON in comparison to the corresponding JOT group (Fig. 4C). The *cas3* expression was considerably elevated in the middle-concentration groups of AFB1 and DON. Besides, its expression was considerably elevated in the low-concentration group of JOT in comparison to the control group and the corresponding single exposure group of DON. However, its expression was considerably reduced in the high-concentration group of JOT in comparison to the corresponding single AFB1 group (Fig. 4D). The expression of *cas9* was surprisingly increased in the high-concentration groups of AFB1 and JOT. Furthermore, its expression was surprisingly elevated in the low-concentration group of JOT (Fig. 4E). The *apaf-1* expression was radically reduced in the low-concentration group of DON. Moreover, its expression was markedly decreased in the low-concentration group of JOT in comparison to the corresponding single AFB1 group. Furthermore, the *apaf-1* expression was also radically reduced in the middle-concentration group of JOT in comparison to the corresponding single DON group (Fig. 4F). The expression of *bax* was outstandingly accelerated in all the single exposure groups of AFB1 and DON. Similar to the *Cu/Zn-sod* expression, the expression of *bax* was not altered in all the JOT exposure groups in comparison to the corresponding single AFB1 or DON groups (Fig. 4G). The expression of *p53* was markedly elevated in the middle- and high-concentration groups of AFB1. Similar to the expressions *of Cu/Zn-sod* and *bax*, the expression of *p53* did not change in all the JOT exposure groups in comparison to the corresponding single exposure groups of AFB1 or DON (Fig. 4H).

3.3.2. Influence on genes involved in immunology system and hypothalamic–pituitary–thyroid (HPT) axis

The *IL-8* expression was enhanced in the middle-concentration group of AFB1 in comparison to the corresponding JOT exposure group. Moreover, its expression was also increased in the high-concentration group of DON (Fig. 5A). The expression of *cc-chem* was prominently enhanced in the

middle- and high-concentration groups of AFB1 in comparison to the control group. Besides, its expression was prominently increased in the low-concentration group of JOT in comparison to the corresponding single DON exposure group. Furthermore, the expression level was prominently enhanced in the high-concentration group of JOT in comparison to the corresponding single exposure group of AFB1 or DON (Fig. 5B). The *cxcl-clc* expression was noteworthily up-regulated in the middle- and high-concentration groups of DON. Also, its expression was noteworthy up-regulated in the high-concentration group of JOT in comparison to the control group and the corresponding single exposure group of AFB1 (Fig. 5C). The expression of *TRα* was conspicuously elevated in the low-concentration groups of DON and JOT. Nonetheless, its expression was not conspicuously altered in all the JOT exposure groups in comparison to the corresponding single exposure groups of AFB1 or DON (Fig. 5D). The expression of *tsh* was observably induced in the high-concentration groups of AFB1 and DON. Similar to the TRα expression, the expression of *tsh* did not observably change in all the JOT exposure groups when comparedwith the corresponding single AFB1 or DON exposure groups (Fig. 5E). The expression of *dio1* was extraordinarily increased in the low-concentration groups of AFB1 and DON. Moreover, its expression was extraordinarily elevated in the middle-concentration group of JOT in comparison to the individual AFB1 exposure group. Contrarily, the expression of *dio1* was extraordinarily reduced in the middle-concentration group of AFB1 (Fig. 5F).

3.3.3. Influence on genes involved in hypothalamic–pituitary–gonadal (HPG) and HPA axes

Similar to the SOD and caspase-9, the *ERα* and *ERβ1* expressions were not manifestly different in all the single and JOT exposure groups in comparison to their corresponding control groups. Additionally, their expressions were not manifestly different in the JOT exposure groups in comparison to their corresponding single exposure groups (Fig. 6A, B). The expression of *cyp17* was enormously enhanced in all the AFB1 and JOT exposure groups (except for the high-concentration groups of AFB1 and JOT). Moreover, its expression was enormously enhanced in the middle-concentration group of DON (Fig. 6C). The expression of *cyp19a* was not drastically altered in all the single exposure groups. Nevertheless, its expression was markedly elevated in the low-concentration group of JOT in comparison to the control group and the corresponding single exposure group of AFB1 or DON. The *cyp19a* expression was remarkably induced in the middle-concentration group of JOT (Fig. 6D). The *crh* expression was sharply elevated in the high-concentration group of AFB1 in comparison to the control group. On the contrary, its expression was sharply decreased in the low-concentration group of JOT (Fig. 6E). The expression of *gr* was markedly elevated in the high-concentration groups of AFB1 and JOT. Its expression was remarkably up-regulated in the middle-concentration group of AFB1 in comparison to the corresponding JOT exposure group. In contrast, its expression was markedly suppressed in the low-concentration group of JOT (Fig. 6F).

3.4. Integrated biomarker response (IBR)

To uncover toxic difference of AFB1, DON and their mixtures with different concentrations, IBR was used to integrate all the determined indicators. Data of zebrafish embryos exposed to mycotoxins at different concentrations were selected to calculate IBR index. Toxicity order of the individual and mixture mycotoxins at different concentration can be compared. The results were represented as star plot (Fig. 7). Overall, AFB1 and JOT exposures exhibited stronger response than DON to the anti-oxidant biomarkers. In contrast, AFB1 and DON elicited the strongest response among all the exposures to apoptotic and detoxification biomarkers, respectively (Fig. 7B and C). Besides, the JOT exposure displayed the strongest response among all the exposures to the endocrine system and immune system biomarkers (Fig. 7D and E).

4. Discussion

Acute toxicity test provides useful information on a wide range of concentrations that can be adopted in the following toxicity testing and the assessment of the therapeutic impacts of toxins [24]. Former reports have displayed that the 4-day LC_{50} value of AFB1 to zebrafish larvae is 0.51 mg a.i.L^{-1}, which is not consistent with our findings [25]. Such a prominent difference might be likely attributed to the different life periods. Moreover, our data showed that AFB1 possessed stronger toxicity to zebrafish in comparison to DON. The determined mycotoxins had prominently different toxicities, which might be attributed to deviations in toxicokinetics and dynamics, such as uptakes, supersession, excretion, or binding to the target sites in *D. rerio* [26]. Therefore, the exposure of AFB1 and DON had latent risks to aquatic animals. Nevertheless, previous investigations on AFB1 and DON have mainly focused on their single toxic effect, while the toxicity of their combinations remains largely unknown.

Because of the natural co-occurrence in the food of mycotoxin, a great deal of attention has been paid to the effects of the mixture exposure [27]. Zebrafish embryos are fertilized ex utero and evolve independently of the mother, making zebrafish embryos an adequate animal model for compound exposure study [28]. Besides, due to its morphological transparency, the zebrafish embryo favors real-time histological observation in vivo [29]. However, more importantly, previous reports have shown that the genetic similarity between humans and zebrafish is more than 80%, and zebrafish has similar signal transduction pathways with suckler [30]. Because of these, zebrafish toxicity studies can serve as an important reference to humans [31]. Consequently, embryonic zebrafish were adopted to evaluate the mixture toxic effect of AFB1 and DON in this study. An additive effect was observed by exposing zebrafish embryos to AFB1 and DON mixtures, indicating that the mixture of AFB1 and DON had stronger mixture toxicity in comparison to their single compounds [19]. Our findings manifested that it was imminently essential to assess the combined toxicity of mycotoxins because the toxicity evaluation of mycotoxins is generally performed barely on single compounds, which might result in an underrated toxic effect in realistic situations.

With the wide existence of mycotoxins in food, it is very necessary to clarify the detoxification mechanism of mycotoxins in humans and how mycotoxins impair and influence human's detoxification mechanism [32]. Our findings indicated that changed CYP 450 and GST activities might contribute to the

toxic action of AFB1 in *D. rerio*, and the elevated GST activity might be the toxic mechanism of DON in the animals. Furthermore, the decreased CarE and GST activities in the high-concentration group of JOT in comparison to the corresponding single exposure group of DON, and the enhanced CYP450 activity in the high-concentration group of JOT might result in the overall additive toxicity of AFB1 and DON [33]. Therefore, all the determined three detoxification enzymes were involved in the detoxification mechanism of mixture toxicity.

As a cell suicide mechanism, apoptosis can get rid of superfluous or undesired cells [34]. The caspase family has a critical function in cell apoptosis, and caspase-3 has been shown as an important executor that is activated downstream in apoptosis pathways [35]. As a transcription factor, the *P53* protein can prevent tumorigenesis via inducing cell cycle arrest and apoptosis [36]. *Apaf-1* is the molecular core of the apoptosome, which is the executioner of mitochondria-dependent apoptosis [37]. Caspase-9 can bind to the apoptosome where it can induce executioner caspases, such as caspase-3 [38]. As a cardinal proapoptotic member of the BCL-2 family, the BCL-2-associated X protein (*bax*) can mediate the critical equilibrium in a cell cycle [39]. We showed that the caspase-3 activity was markedly induced in the AFB1 exposure, while no prominent change in caspase-9 was observed, which was not in line with the gene expression pattern. AFB1 apparently stimulated the expressions of *cas3*, *cas9*, *bax*, and *P53* at the mRNA level, showing that this mycotoxin caused apoptosis via activating *P53*, which directly triggered the expression of gene encoding the proapoptotic protein (*bax*), then induced the critical executor of apoptosis (*cas3*) activation and subsequently resulted in cell apoptosis [35]. Moreover, the expressions of *cas3*, *cas9*, and *apaf-1* were markedly altered in the JOT group, implying that regular apoptosis was influenced by the co-exposure of AFB1 and DON.

Environmental stressors can immediately stimulate oxidative stress via a complex physiological process, and oxidative stress occurs when the level of reactive oxygen species (e.g., $O_2{\cdot}^-$, H_2O_2, ·OH, etc.) is higher than the scavenging activities of antioxidants [40]. The antioxidants, including SOD, CAT, and POD, can protect the cells from oxidative stress through their important role in the defense mechanism [41]. SOD can scavenge $O_2{\cdot}^-$and catalyze it to O_2 and H_2O_2 [34]. The H_2O_2 generated is further detoxified via CAT and other enzymes, such as POD. The changes in their contents may indicate the extent of damage to the organism [42]. Both SOD and CAT activities in the single mycotoxin exposure groups were not markedly altered, indicating that the determined concentration of single mycotoxin was insufficient to induce prominent oxidative stress. However, a prominent reduction was found in the high-concentration group of JOT in comparison to the corresponding single exposure group of DON, which could be attributed to an overwhelmed antioxidant capacity [43]. LPO is a reliable oxidative stress indicator since it plays a critical role in denoting cell damage. MDA is the primary by-product of LPO, and an elevation in its content incarnates the degree of the cell damage caused by free radicals [44]. However, the MDA level was reduced in the AFB1 and JOT exposure groups, potentially suggesting that LPO was reduced by antioxidants. The expressions of anti-oxidative genes are necessary for evaluating antioxidant capacity [35]. In our current study, AFB1 and DON exposures up-regulated the expressions of *Mn-sod* and *Cu/Zn-sod* at the mRNA level, which was not consistent with the SOD activity, suggesting that SOD was

primarily modulated at the protein level. The increased expressions of *Mn-sod* and *Cu/Zn-sod* may induce the SOD activity and eliminate the superoxide anion radical caused by the exposure of AFB1 and DON. Besides, the up-regulation of *gpx* at the mRNA level could also activate the gpx activity. Such discrepancy between antioxidant activity and gene expression might also be attributed to the presence of multiple gene copies in the species, a time-lag, and post-transcriptional modifications [45].

THs play an important role in the growth and development of fish and the thyroid endocrine system, while the HPT axis can regulate the thyroid system [46]. In the current study, the levels of T3 and T4 were markedly changed after exposure to AFB1 or DON, and their contents were also distinctly altered in the JOT exposure groups. Besides, the T4 level was markedly increased in the JOT exposure. The expression of the *TRa* gene was dramatically enhanced in the low-concentration group of DON, indicating that a mechanism negatively responded to the reduced T4 level [47]. On the contrary, a marked up-regulation of the *TRa* gene was found in the low-concentration group of JOT, implying that there was a positive feedback mechanism because of the induced T4 level [48]. Noticeably increased expression of *tsh* was found in the high-concentration groups of AFB1 and DON in comparison to the control group. Iodothyroinine deiodinases (Dio) possess an important function in modulating the circulation of peripheral TH levels and maintaining the ratios of T4 and T3 in vertebrates [49]. *Dio1* affects iodine recovery and TH degradation. The expression of *dio1* was negatively associated with T3 and T4 levels in fish upon exposure to AFB1, DON, and their mixture. Consequently, the changes in T3 and T4 contents in zebrafish indicate the maladjustment of THs [50]. The expressions of thyroid-associated genes were also considerably changed, indicating that AFB1, DON, and their mixture were thyroid disruptors in embryonic zebrafish.

VTG is considered a biomarker of estrogen-associated endocrine disorders, and the generation of VTG is regulated via 17β-estradiol activation of estrogen receptors (ERs) [51, 52]. The VTG content was obviously reduced in the AFB1 and DON exposure groups, indicating that the two mycotoxins had the anti-estrogenic effect. Conversely, the VTG content was increased in the JOT exposure group, implying that the mixture of AFB1 and DON possessed the estrogenic effect [53]. HPG axis modulates sex hormones, which are tightly associated with procreation in fish [54]. Surprisingly up-regulated expressions of *cyp17* and *cyp19a* were found in the AFB1, DON, or JOT group. Besides, the *cyp19a* expression was remarkably up-regulated in the low-concentration group of JOT implies that AFB1, DON, and their mixture could impair the reproduction of zebrafish.

The main function of the HPA axis is to modulate the adaptive stress response of organisms [55]. The expressions of *crh* and *gr* were noticeably varied in most of AFB1 and JOT exposure groups, implying that AFB1 and its combination with DON could impact the development of zebrafish. Environmental pollutants can elevate or reduce the expressions of immune-related genes [56]. Our present study exhibited that the single and JOT exposures increased the expressions of *IL-8*, *cc-chem*, and *cxcl-cic* at the mRNA level. This finding might imply that latent tissue damage triggered by AFB1, DON, and their blend provoked the up-regulation of the investigated cytokines in this study [57]. Moreover, AFB1, DON, and their mixture might impair the immune function and elevate the susceptivity to toxicants for fish.

A test of multiple toxicities in zebrafish is helpful in comprehensively understanding the toxic mechanisms of environmental pollutants [36, 52]. To comprehensively assess the mixture mechanisms of AFB1 and DON in fish, we attempted to assess the toxic effects of mixture mycotoxins on oxidative stress, immunotoxicity, cell apoptosis, and endocrine system in the early developmental stage of zebrafish at various endpoints. Our data provided solid evidence on the mixture toxic mechanism of AFB1 and DON in aquatic organisms. In the present study, the altered enzyme activity reflected the extent of cellular damage. Nevertheless, different biochemical enzymes have different sensitivity to mycotoxins. To validate this hypothesis, further study is still required to evaluate the changes of these enzymes at the protein level. The zebrafish can adapt to various stimuli induced by exogenous mycotoxins via a potential mechanism by up/down-regulating genes. Therefore, it is indispensable to evaluate the mechanism underlying the different alternations in immune-associated genes.

5. Conclusions

AFB1 exhibited greater acute toxicity than DON to the embryos of *D. rerio* and the mixture presented an additive effect. The levels of CAT, caspase-3, and T4 were markedly varied in most single and mixture groups. The expression levels of four genes (cas3, apaf-1, cc-chem, and cyp19a) associated with oxidative stress, cellular apoptosis, immune system, and endocrine system varied markedly in the mixture group in comparison to the corresponding single group. A comprehensive assessment of toxic effects of AFB1, DON, and their mixture using multiple endpoints offered valuable insights into the total toxicity induced by single and mixture mycotoxins in zebrafish as well as its potential mechanism.

Abbreviations

AFB1	Aflatoxin B1
DON	Deoxynivalenol
ROS	Reactive Oxygen Species
MDA	Malonaldehyde
LPO	Lipid Peroxidation
SOD	Superoxide Dismutase
CAT	Catalase
POD	Peroxidase
CYP450	Cytochrome P450
CarE	Carboxylesterase
GST	Glutathione-S-Transferase
VTG	Vitellogenin
THs	Thyroid Hormones
T3	Triiodothyronine
T4	Thyroxine
LC_{50}	Median Lethal Concentration
JOT	The Combined Exposure of AFB1 and DON
L	Low Concentration
M	Middle Concentration
H	High Concentration

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yanhua Wang: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. Qiang Wang: Conceptualization, Data curation, Formal analysis, Writing - original draft, Project administration. Chun Ji: Conceptualization, Investigation, Methodology, Resources. Xiaoxuan Guo: Conceptualization, Investigation, Methodology, Resources. Guiling Yang: Conceptualization, Data curation, Formal analysis, Writing - original draft. Dou Wang: Writing - original draft, Investigation, Methodology, Resources. Hongbiao Weng: Supervision, Funding acquisition. Yongzhong Qian: Conceptualization, Supervision, Funding acquisition, and Project administration. Chen Chen: Conceptualization, Writing - original draft, Supervision, Funding acquisition, Project administration, Writing - review & editing.

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Figures

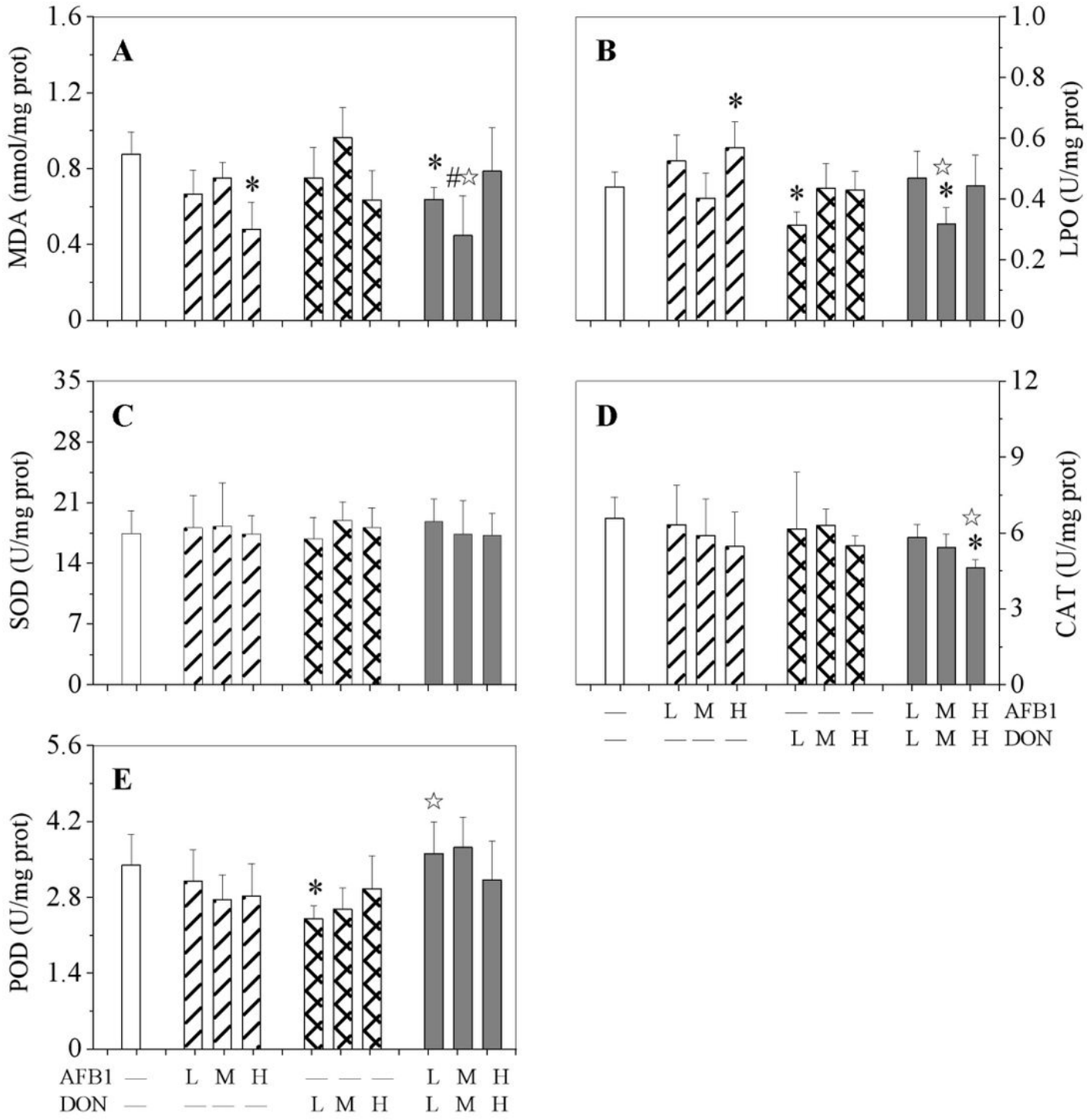

Figure 1

The oxidative reactions of fish embryos administrated with AFB1, DON and their combinations. Data are presented as the means ± standard deviation of three triplicates. * p< 0.05, substantial alteration by comparison with the control; # p< 0.05, substantial alteration by comparison with the AFB1 administration group at counterpart concentration; ☆p< 0.05, substantial alteration by comparison with

the DON administration group at counterpart concentration. AFB1 = aflatoxin B1; DON = deoxynivalenol; L = low concentration; M = middle concentration; H = high concentration.

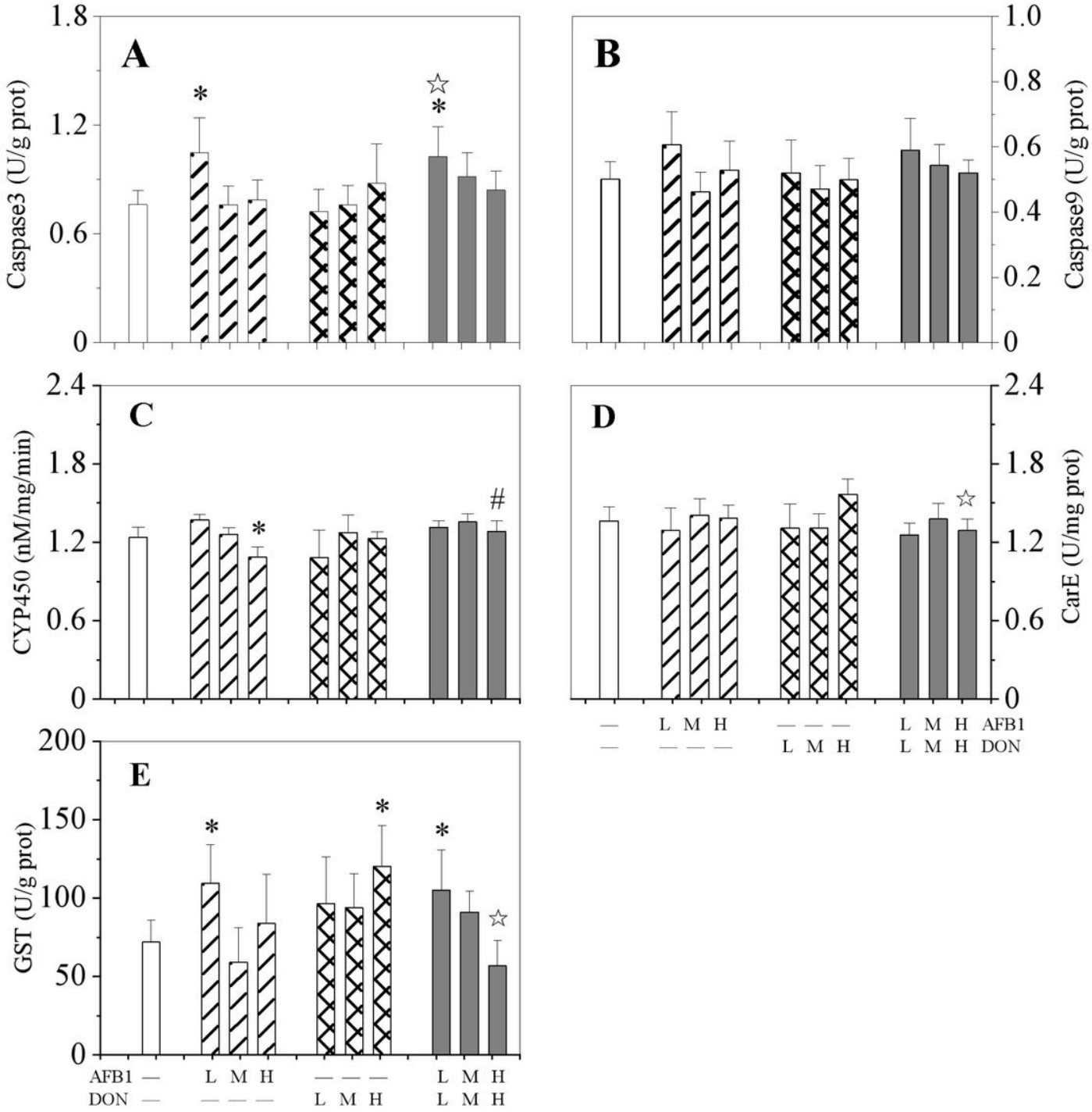

Figure 2

The apoptotic and detoxification enzyme activities of fish embryos administrated with AFB1, DON and their combinations. Data are presented as the means ± standard deviation of three triplicates. * p< 0.05, substantial alteration by comparison with the control; # p< 0.05, substantial alteration by comparison with the AFB1 administration group at counterpart concentration; p< 0.05, substantial alteration by

comparison with the DON administration group at counterpart concentration. AFB1 = aflatoxin B1; DON = deoxynivalenol; L = low concentration; M = middle concentration; H = high concentration.

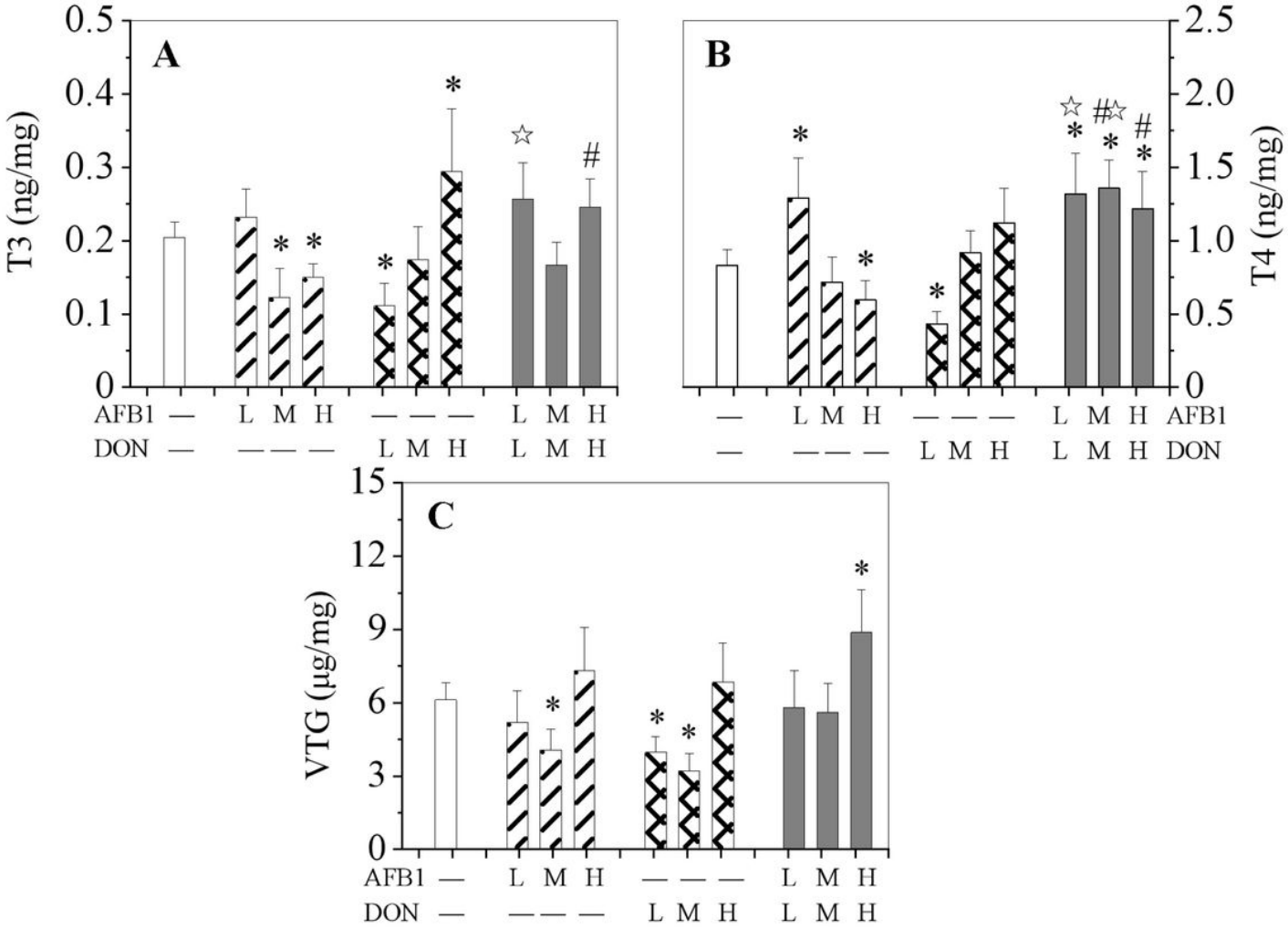

Figure 3

Thyroid hormones and VTG levels of fish embryos administrated with AFB1, DON and their combinations. Data are presented as the means ± standard deviation of three triplicates. * p< 0.05, substantial alteration by comparison with the control; # p< 0.05, substantial alteration by comparison with the AFB1 administration group at counterpart concentration; ☆p< 0.05, substantial alteration by comparison with the DON administration group at counterpart concentration. AFB1 = aflatoxin B1; DON = deoxynivalenol; L = low concentration; M = middle concentration; H = high concentration.

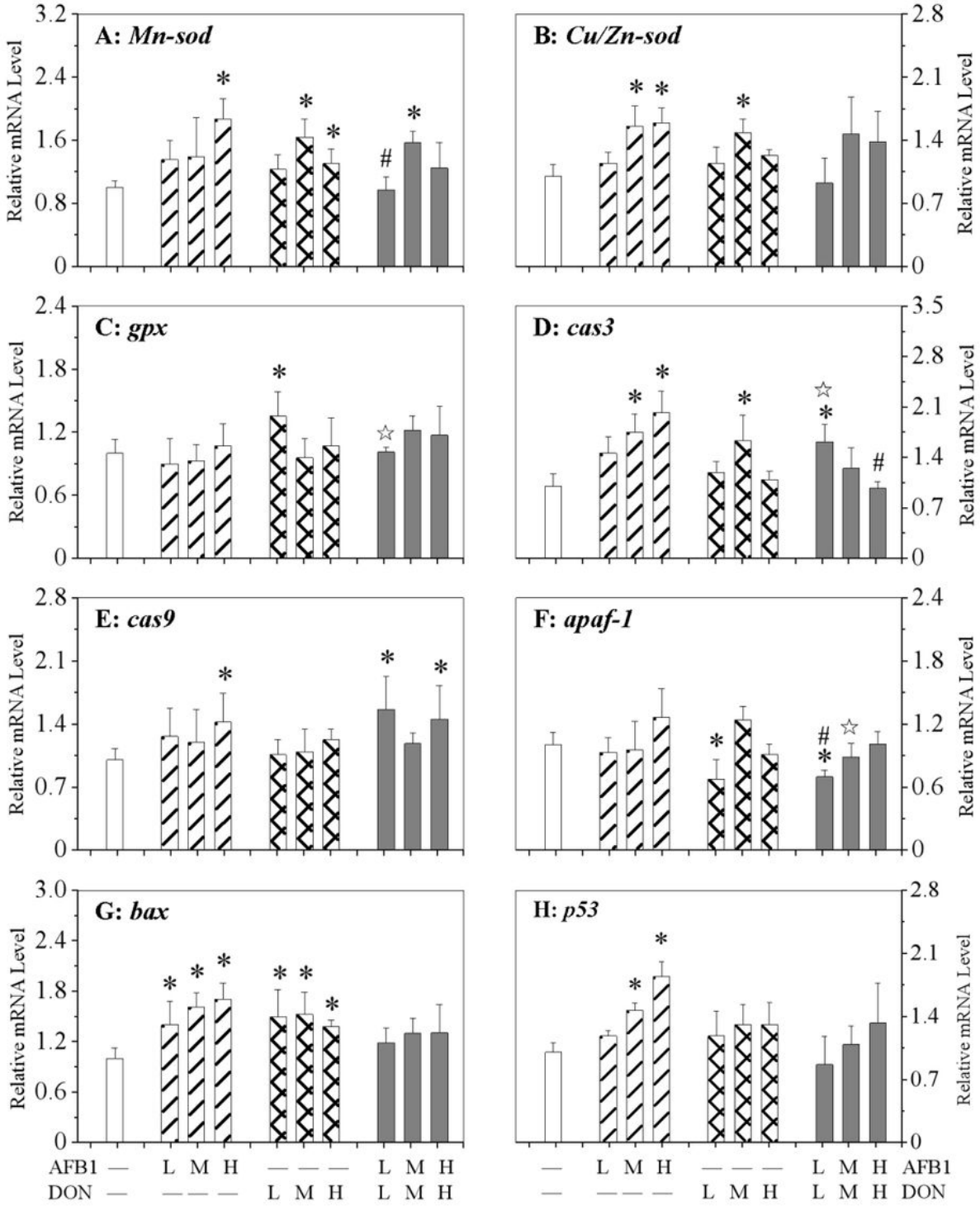

Figure 4

Influences on transcription levels of genes associated with the anti-oxidative systems and cell apoptosis of fish embryos administrated with AFB1, DON and their combinations. Data are presented as the means ± standard deviation of three triplicates. * $p < 0.05$, substantial alteration by comparison with the control; # $p < 0.05$, substantial alteration by comparison with the AFB1 administration group at counterpart concentration; □$p < 0.05$, substantial alteration by comparison with the DON administration group at

counterpart concentration. AFB1 = aflatoxin B1; DON = deoxynivalenol; L = low concentration; M = middle concentration; H = high concentration.

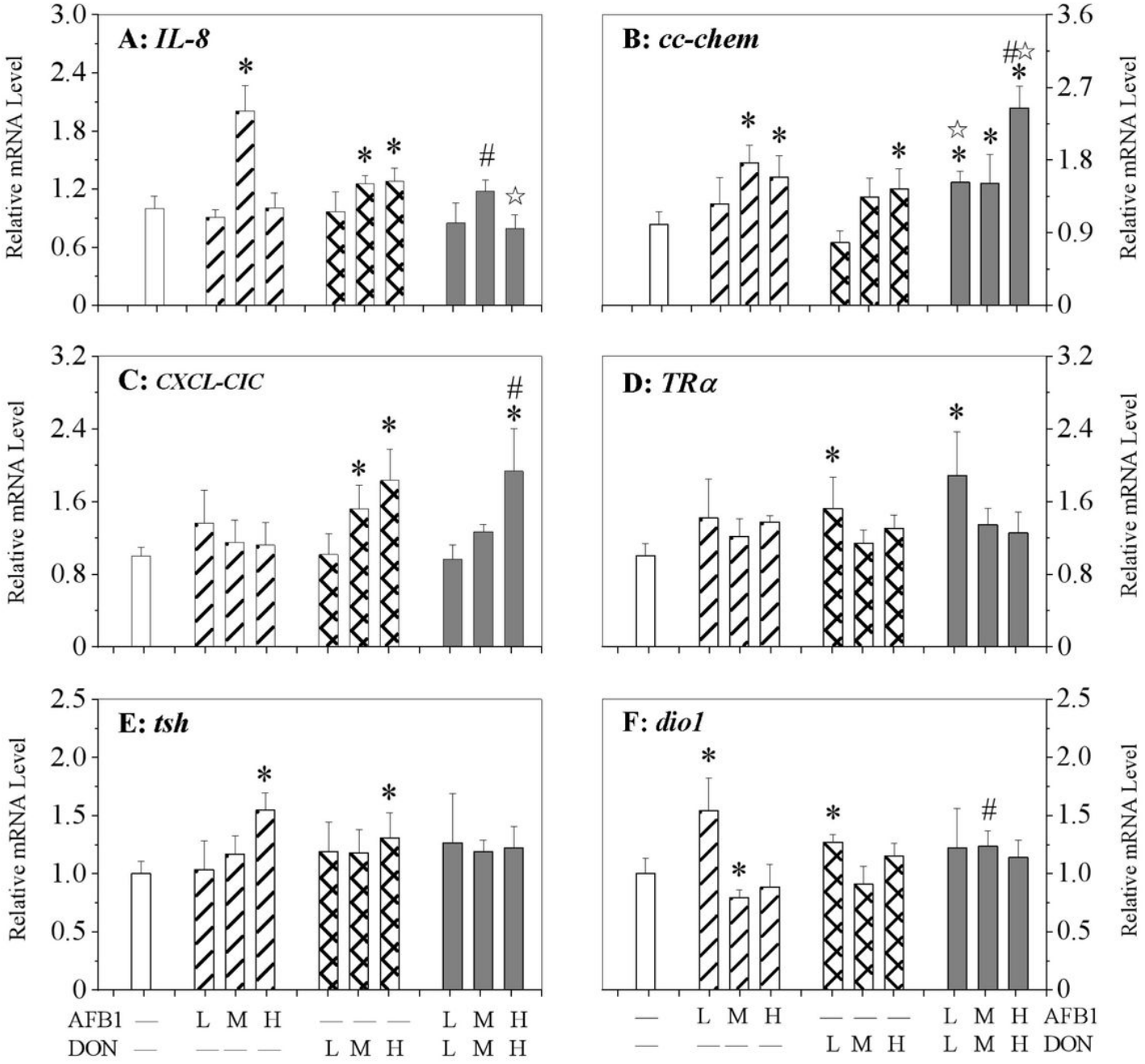

Figure 5

Influences on transcription levels of genes associated with immunology system and HPT axis of fish embryos administrated with AFB1, DON and their combinations. Data are presented as the means ± standard deviation of three triplicates. * p< 0.05, substantial alteration by comparison with the control; # p< 0.05, substantial alteration by comparison with the AFB1 administration group at counterpart concentration; ☆p< 0.05, substantial alteration by comparison with the DON administration group at

counterpart concentration. AFB1 = aflatoxin B1; DON = deoxynivalenol; L = low concentration; M = middle concentration; H = high concentration.

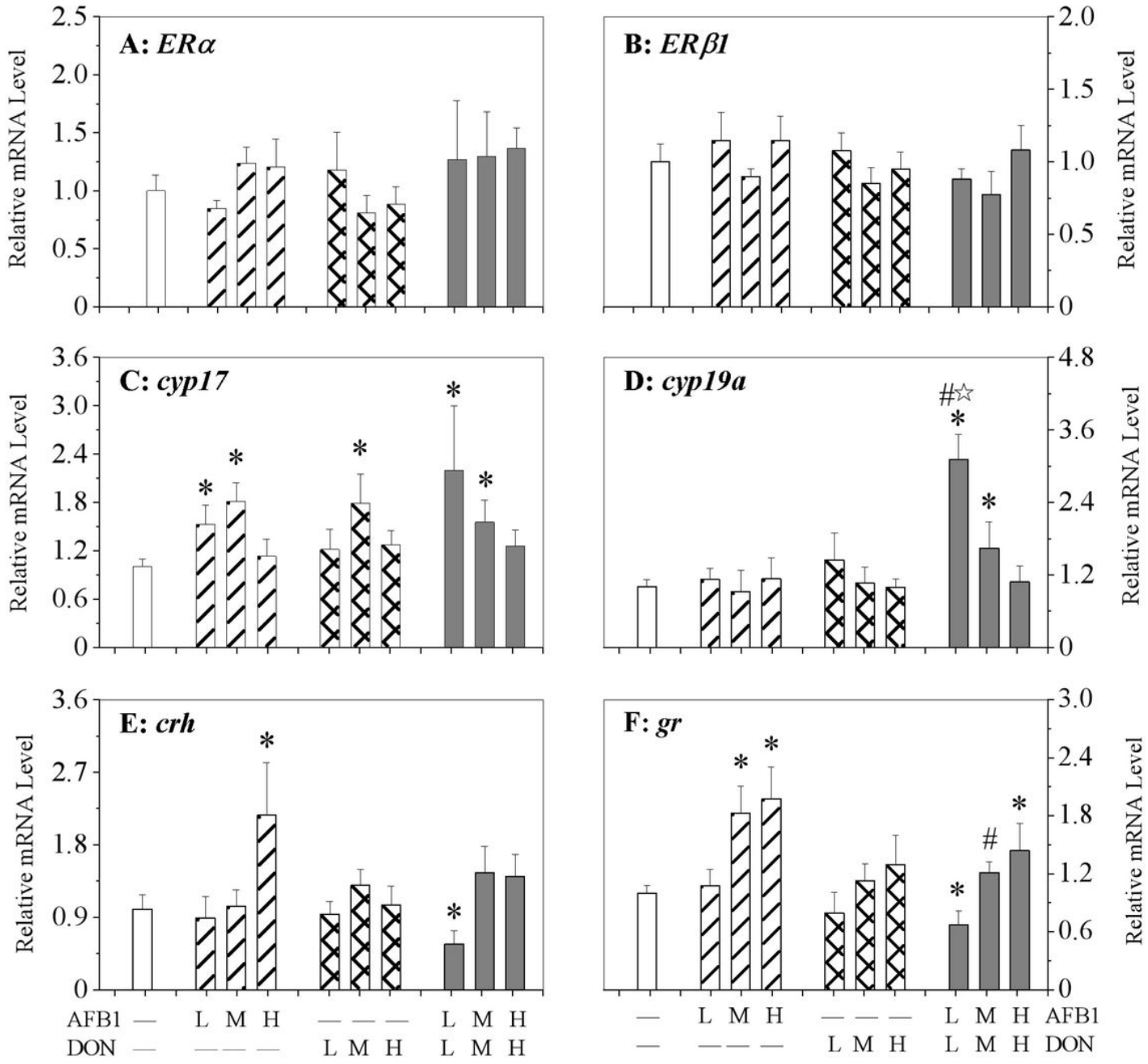

Figure 6

Influences on transcription levels of genes associated with the HPG and HPA axes of fish embryos administrated with AFB1, DON and their combinations. Data are presented as the means ± standard deviation of three triplicates. * p< 0.05, substantial alteration by comparison with the control; # p< 0.05, substantial alteration by comparison with the AFB1 administration group at counterpart concentration; ☆p< 0.05, substantial alteration by comparison with the DON administration group at counterpart

concentration. AFB1 = aflatoxin B1; DON = deoxynivalenol; L = low concentration; M = middle concentration; H = high concentration.

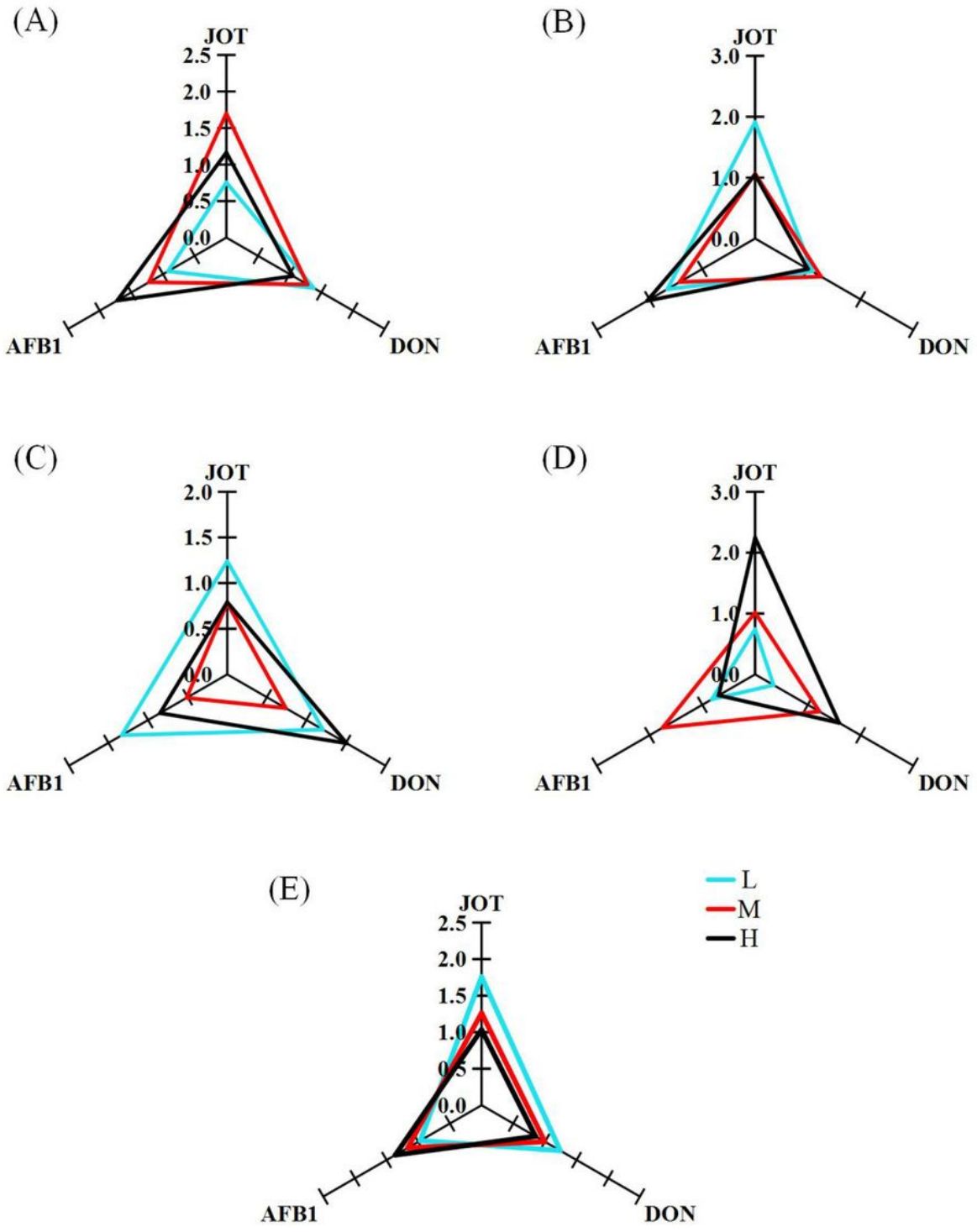

Figure 7

Star plots of integrated biomarker response of different parameters. (A): IBR index of anti-oxidant biomarkers; (B): IBR index of apoptotic biomarkers; (C): IBR index of detoxification biomarkers; (D): IBR index of endocrine system biomarkers; (E): IBR index of immune system biomarkers. AFB1 = aflatoxin B1;

DON = deoxynivalenol; JOT = Joint treatment of AFB1 and DON; L = low concentration; M = middle concentration; H = high concentration.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalMaterialsforAFB1DON.doc