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Combined toxic effects of T-2 toxin and propiconazole on the early life stages of zebrafish (*Danio rerio*)

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Abstract

Background Agricultural products are frequently co-contaminated by mycotoxins and pesticides. Most studies have only focused on the single toxicities of these chemicals, while their combined effects are neglected. Therefore, we investigated the combined toxic effects of T-2 toxin (T-2) and propiconazole (PRO) on zebrafish (*Danio rerio*).

Results Our data exhibited that T-2 had higher toxicity to embryonic fish (96-h LC_{50} value of 0.39 mg a.i. L^{-1}) than PRO (96-h LC_{50} value of 17.16 mg a.i. L^{-1}). The mixture of T-2 and PRO showed an acute synergistic effect on zebrafish. Meanwhile, indicators associated with oxidative stress (SOD, *Mn-sod,* and *cat*) displayed significant variations in most exposures to T-2 and PRO mixtures (MTP) compared with the single exposures. The expressions of apoptosis-related genes *cas3* and *cas9* were also substantially elevated in the high-dose MTP exposure compared with the corresponding T-2 exposure. Besides, the expressions of endocrine system-related genes (*TRβ*, *tsh*, *crh*, *cyp19a,* and *vtg1*) were markedly varied in most MTP exposures compared with the corresponding single exposures. Our present results suggested that the mixture of T-2 and PRO could cause enormous effects on oxidative stress, cellular apoptosis, and the hypothalamic–pituitary–thyroid/hypothalamic–pituitary–gonadal (HPG/HPT) axis of zebrafish.

Conclusions Our results provided new insights into the development of combined pollution standards for agricultural products. Taken together, the impact of the combined effects could be considered and regulated as priorities.

Keywords Co-exposure, Mycotoxin, Toxic mechanism, Synergistic action

Background

Mycotoxins and pesticides are common contaminants in agricultural products, which are widely present in almost all aspects of the production, processing, and storage processes [1]. These chemicals can cause food pollution and exert negative effects on animal and human health [2]. Previous surveys have indicated that about 25% of global agricultural products are polluted by mycotoxin each year, and long-term exposure to them presents a substantial risk to human health [3, 4]. Pesticides are used to achieve high yields and high quality in agricultural products, and the toxic effects caused by their residues have been reported frequently [5]. Only very few studies have reported the combined effects of pesticides and mycotoxins on animals. Aflatoxin and cypermethrin have been shown to increase mortality in rats after combined exposure compared with their individual exposures [6]. Similarly, patulin, in combination with chlorpyrifos has been reported to synergistically induce hepatotoxicity in AML12 mouse liver cells [7]. Nonetheless, most

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studies merely focus on the toxicities of pesticides or mycotoxins individually [8–10]. Considering that mycotoxins and pesticides are often present as complex mixtures in agricultural products, it is crucial to investigate their combined toxicities to human health [11].

As a secondary metabolite generated by the fungus *Fusarium oxysporum,* T-2 toxin (T-2) is one of the most toxic food-derived toxins [12, 13]. It has a high heat resistance and non-volatile properties and will not be inactivated during food processing [14, 15]. A previous investigation has demonstrated that T-2 can cause reproductive organ atrophy and a decline in testicular function in mice [16]. In addition, exposure to T-2 increases reactive oxygen species (ROS) production and induces mitochondrial dysfunction in the neuronal cells, suggesting that T-2 has neurotoxicity effects [17]. The increase in leukocyte number and the decrease in bactericidal activity indicate that the T-2 can affect both non-specific cellular and humoral immunity in rainbow trout (*Oncorhynchus mykiss*) [18]. Ingestion of T-2-contaminated food not only can cause damage to tissues and organs, but also is closely related to degenerative joint diseases, such as Kashin–Beck disease (KBD) [19–21]. Propiconazole (PRO) is a triazole fungicide commonly used on corn, wheat, and vegetables worldwide [22]. It is characterized by a long half-life and easy diffusion in the environment [23, 24]. It has been confirmed that PRO can cause neurobehavioral toxicity in mammals [25, 26]. PRO can also disturb the endocrine system and reproductive function of zebrafish [27, 28]. Previous examinations on T-2 and PRO have mainly focused on their individual effects, while their combined toxicities are poorly documented [29, 30].

Recent evidence has exhibited that T-2 and PRO often co-exist in the same agricultural products [31]. Therefore, increasing attention has been paid to their joint toxic effect on human health [32]. Zebrafish (*Danio rerio*) have the advantages of a short reproductive cycle and a high spawning capacity, making them an ideal model in the field of toxicology [33]. Moreover, its genome is up to 87% homologous relative to the human genome, and the biosynthetic pathway is similar to that of humans [34]. When zebrafish are exposed to contaminants, they can respond physiologically, with gene expression, and behavior [35]. Therefore, the results obtained from zebrafish model research have high compatibility with human detection data and have great reference value [36, 37]. In this context, we determined the changes in enzymatic activity and gene expression when *D. rerio* were exposed to the mixture of T-2 and PRO. The data obtained would be helpful in understanding the overall toxicity of T-2 and PRO mixtures for zebrafish and promote the further exploration of the underlying hazards in other co-existing mycotoxins and pesticides for agricultural products. In addition, EU (EC) 396/2005 stipulates that the synergistic effects of multiple pesticides should be considered when developing pesticide MRL (maximum residue limit) standards [38]. Therefore, combined toxicity assessment of mycotoxins and pesticides played a vital role in developing more realistic agricultural product quality standards and monitoring guidelines.

Materials and methods

Chemicals

T-2 (purity ≥ 98%, CAS number: 21259-20-1) was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). PRO (purity ≥ 99%, CAS number: 60207-90-1) was purchased from Hubei Marvel Co., Ltd. (Wuhan, China). The stock solutions of chemical were formulated in dimethyl sulfoxide (DMSO, purity > 99.9%; Amresco, Solon, OH, USA) and 10% Tween-80 (Wt: Vol). All stock solutions were diluted with reconstituted water. The composition of reconstituted water was 2 mmol/L Ca^{2+},0.5 mmol/L Mg^{2+}, 0.75 mmol/L Na^{+}, and 0.074 mmol/L K^{+} [39].

Collection of the zebrafish embryos

Adult zebrafish of the AB strain were purchased from the Zebrafish Research Center of China (Wuhan, China) and were maintained in a recirculation system at 27 ± 1 ℃ with a 14/10-h light/dark photoperiod. The fish were fed a commercial brine shrimp flake twice daily. Mature female and male zebrafish (ratio of 1:2) were placed in the egg-laying boxes to obtain embryos overnight. Light stimulation was initiated the next morning, and the eggs were collected 30 min later. Normal embryos were selected for subsequent experiments by microscopic observation. All procedures were authorized by the Independent Animal Ethics Committee of the Zhejiang Academy of Agricultural Science.

Acute toxicity test

Individual toxicity assay

Acute toxicity assay of individual chemicals was performed according to the previous experimental protocol and OECD test guideline 236 [40]. In order to maintain the appropriate concentration of chemical and water quality, the semi-static method was adopted. All exposure solutions were refreshed every 24 h. Healthy embryos at 3 h post-fertilization (hpf) were placed into 96-well plates, and each well contained one embryo and 0.2 mL of exposure solution. Reconstituted water was set to the blank control. Six different concentrations with a geometrical ratio that caused 10%-90% mortality according to the results from pre-trials were set for T-2 and PRO exposures, respectively. Each concentration consisted of

three replicates. Each replicate contained 24 embryos. Mortality was recorded after 96 h of exposure.

Mixture toxicity assay

The interaction mode of T-2 and PRO was assessed. Significant level of mean separation ($P < 0.05$) was set based on non-overlap between the 95% confidence interval of two LC_{50} values. Briefly, zebrafish embryos at 3 hpf were exposed to T-2 and PRO mixtures at a fixed equitoxic constant mixture ratio with serial dilutions based on the determined individual LC_{50} values [41]. The other procedures of the equitoxic assay were similar to those of the individual assay.

The combined toxicity was evaluated according to the additive index method of Markings [42]. The formula is as follows:

$$S = (Am/Ai) + (Bm/Bi).$$

S is the sum of the additive effect of biotoxicity; Ai and Bi are the LC_{50} values of toxicity of the individual A and B toxicants, respectively; and Am and Bm are the LC_{50} values of A or B in the mixture, respectively. When $S \leq 1$: AI = $1/S - 1.0$; when $S > 1$: AI = 1.0-S. The summation index method (AI) was used to assess the combined effects of pesticides: AI > 0.25 for synergistic effects, AI ≤ − 0.2 for antagonistic effects, and − 0.2 < AI ≤ 0.25 for additive effects [43].

Cell- and gene-level determinations

Exposure protocols

Low, middle, and high concentrations were set according to 1/320, 1/80, and 1/20 of 96-h LC_{50} of T-2 and PRO single concentrations, respectively. The low, middle, and high concentrations of the mixture were combinations of T-2 and PRO (MTP) at the low, middle, and high concentrations, respectively. That is, the nominal concentration of T-2 was 1.2 μg a.i. L^{-1}, 4.8 μg a.i. L^{-1} and 19.5 μg a.i. L^{-1}; the nominal concentration of PRO was 53.6 μg a.i. L^{-1}, 214.5 μg a.i. L^{-1} and 858 μg a.i. L^{-1}; the nominal concentration of MTP was (1.2 + 53.6) μg a.i. L^{-1}, (4.8 + 214.5) μg a.i. L^{-1}, (19.5 + 858) μg a.i. L^{-1}.

Final DMSO concentrations were 0.05% in the exposure solution. The use of 0.05% DMSO as a solvent control in the pre-experimental stage did not significantly differ in enzyme activity and gene expression compared to the blank control. Therefore, reconstituted water was used as blank control in this study.

Sample collection

Normally developed embryos (3 hpf) were chosen and placed into crystal dishes containing 500 mL of solution, with about more than 300 embryos in each dish as a replicate. Three replicates were given for each concentration. Dead zebrafish have been removed immediately from the exposure solution. The exposed embryos were placed in an incubator at 27 ± 1 °C for 7 days with a light–dark cycle of 14 h:10 h. In order to maintain the concentration of chemical and water quality, the chemical solutions were refreshed every 24 h in these experiments. After the exposure, zebrafish from each treatment group were collected, 250 fish were designated for determining enzyme activity, and 50 fish were used for RNA extraction. The captured zebrafish were kept in a freezer at − 80 °C.

Cell-level determinations

Briefly, 250 fish were placed into a 2.0-mL centrifuge tube at 7 dpf. Each tube was homogenized (1:10, w/v) using 1 mL PBS (pH 7.4) and centrifuged at 12,000 rpm for 15 min at 5 ℃. After centrifugation, the supernatant was transferred into a new tube, and the biomarker assay was performed immediately.

The activities of SOD, CAT, CYP450, GST, caspase 3, and CarE were measured by respective commercial kits (Shanghai Sangon Biotech Co., Ltd., Shanghai, China) based on the manufacturer's instructions. The content of MDA was also detected using reagent kits from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). In addition, the content of VTG was measured by adopting enzyme-linked immunosorbent assay (ELISA) kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). Protein concentration was determined by a BCA method (Beyotime Biotech Co., Ltd., Shanghai, China). The data of all the biomarkers were normalized to total protein content.

Gene expression analysis

The tested genes, including detoxification-related genes, endocrine disruption-related genes, and immune-related genes, were detected by quantitative real-time PCR as previously described [44]. Briefly, 50 larvae at 7 dpf were used for RNA extraction. Total mRNA was extracted from each sample using the TransZol reagent (Transgen Biotech Co., Ltd., Beijing, China). The first-strand cDNA was synthesized with a vazyme kit (Vazyme Biotech Co., Ltd., Nanjing, China). *β-Actin* was adopted as the housekeeping gene. Sequences of gene-specific primers were selected according to previous studies [45–48]. The primer sequences of genes are shown in Additional file 1: Table S1. The relative expression levels of the target genes were calculated by adopting the $2^{-\Delta\Delta Ct}$ method [49].

Chemical analysis

To verify the actual, T-2, PRO and their mixture exposure solution were determined at the start (0 h) and before water renewal (24 h) during the experimental period. Every concentration has three replicates. The concentration of exposure solution was detected by

liquid chromatography–tandem mass spectrometry (SHIMADZU, LC–MS-8050) with a mobile phase composed of an aqueous solution (5 mmol/L ammonium formate (A) and methanol (B). The separation was conducted using a gradient elution program as follows: 0–0.5 min, 95% A; 0.5–2.0 min, 5% A; 2.0–5.0 min, 5% A; 5.0–5.1 min, 95% A; 5.1–7.0 min, 95% A. Mass spectrometric detection was conducted in positive electrospray ionization (ESI) with multiple reaction monitoring (MRM) modes. For T-2, and the product ions were *m*/*z* 484.4/245.3 for quantization and *m*/*z* 484.4/215.5 for confirmation with collision energies of 27 and 25 eV, respectively. For PRO, the product ions were m/z 172.9/145.0 for quantization and m/z 172.9/74 for confirmation with collision energies of 16 and 11 eV, respectively. Analysis results revealed that the deviations between the nominal and actual concentrations of T-2, PRO, and their mixtures were less than 20%. Therefore, the nominal concentration was used as the actual concentration in the present study.

Statistical analysis

The acute toxicities of mycotoxin and pesticides to zebrafish were analyzed according to the procedure developed by Chi [50]. Data were expressed as means ± standard error of the mean (SEM). All data were statistically analyzed using the SPSS 18.0 software. One-way analysis of variance (ANOVA) for multiple groups and Dunnett's post hoc comparison for two treatments were conducted to compare the data. In all cases, $P < 0.05$ was considered statistically significant. The ΔCt method of the reference gene was used to calculate the expression of interest genes, and performed logarithmic conversion. The housekeeping gene (*β-actin*) was applied relative to Ct Q. Statistical analysis of the qPCR with $2^{-\Delta\Delta Ct}$ method [49]. Gene data of the heatmap were transformed for Log10 and homogenized.

Results

Determinations of single and joint toxicities

Table 1 presents the acute toxicity of T-2 and PRO in zebrafish embryos. Results exhibited that the 96-h LC_{50} value of T-2 and PRO was 0.39 mg a.i. L^{-1} and 17.16 mg a.i. L^{-1}, respectively, indicating that T-2 had higher toxicity to zebrafish embryos compared with PRO. To explore the combined effect, we also tested the LC_{50} values of T-2 and PRO in their mixture after 96 h of exposure. Based on the LC_{50} values of T-2 and PRO individually and in the mixture, the mixture of T-2 and PRO exerted acute synergetic toxicity to zebrafish with an AI value of 0.63. The mortalities of every chemical to zebrafish larvae, individually and combined, are presented in Additional file 1: Table S2.

Determinations of biochemical level

Determinations of oxidative stress

The SOD activity was markedly increased in all the individual exposures (except for the high-dose PRO exposure) compared with the control group. By contrast, a remarkable decrease was found in the low-dose MTP exposure compared with the corresponding T-2 and PRO exposures. Its activity was also prominently weakened in the middle- and high-dose MTP exposures compared with the corresponding T-2 exposures (Fig. 1A). The MDA content was dramatically reduced in all the individual exposures (except for the low-dose PRO exposure) and the high-dose MTP exposure compared with the control group. In contrast, a significant enhancement was noticed in the middle-dose MTP exposure compared with the control group. Its content was also dramatically elevated in the middle-dose MTP exposure compared with the individual exposures (Fig. 1B). The CAT activity was steeply increased in the low- and middle-dose T-2 exposures, as well as the high-dose PRO exposure, compared with the control group. However, a marked weakening was detected in the low-dose MTP exposure compared with the corresponding T-2 exposure (Fig. 1C).

Apoptotic and detoxification enzyme activities

The caspase 3 activity was raised in all MTP exposures compared with the control group. A rise was also registered in the low- and middle-dose MTP exposures compared with the corresponding T-2 exposures (Fig. 2A). The activity of CYP450 was inhibited in all the T-2 and PRO exposures (except for the middle-dose PRO

Table 1 Individual and mixture toxicities of T-2 toxin and propiconazole to the larvae of *Danio rerio*

LC_{50} (95% FL)[a] mg a.i. L^{-1}		LC_{50} (95% FL)[b] mg a.i. L^{-1}		AI[c] value
T-2 toxin	Propiconazole	T-2 toxin	Propiconazole	
0.39 (0.35 ~ 0.44)	17.16 (14.64 ~ 21.45)	0.12 (0.073 ~ 0.17)	5.28 (3.21 ~ 7.48)	0.63

[a] The LC_{50} (95% confidence limit) for T-2 toxin or propiconazole individually

[b] The LC_{50} (95% confidence limit) for T-2 toxin or propiconazole in the mixture

[c] AI additive index

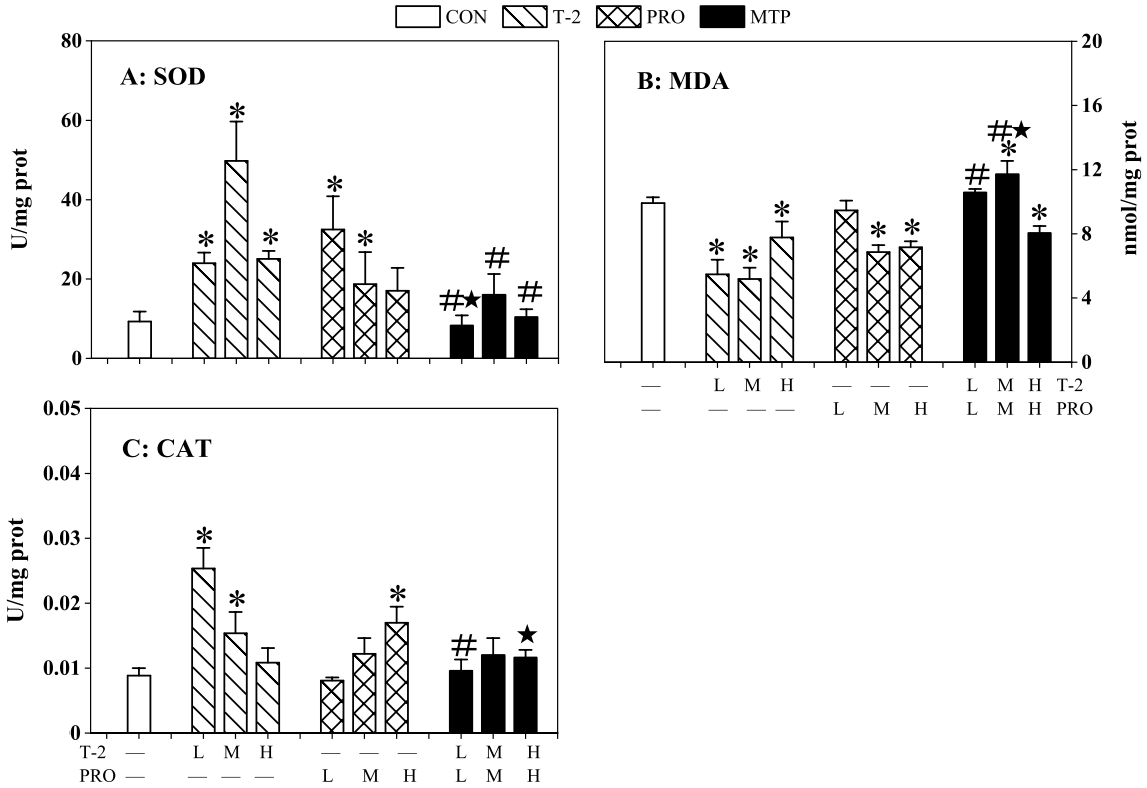

Fig. 1 The oxidative responses in zebrafish treated with T-2 PRO and their combinations. **A** SOD, **B** MDA, **C** CAT. Each bar represents the mean ± standard deviation (n = three replicates consisting of a group of 250 fish). * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding T-2 exposure; ★$p < 0.05$ significant difference relative to the corresponding PRO exposure. *CON* control, *T-2* T-2 toxin, *PRO* propiconazole, *MTP* the mixture of T-2 and PRO. *L* low concentration; *M* middle concentration, *H* high concentration

exposure) compared with the control group. Contrarily, its activity was noticeably increased in the middle-dose MTP exposure compared with the corresponding PRO exposure (Fig. 2B). The activity of CarE was considerably stimulated in the middle-dose T-2 exposure compared with the control group. Nevertheless, a considerable decrement was monitored in the middle-dose MTP exposure compared with the corresponding T-2 exposure (Fig. 2C). The GST activity was enormously elevated in all the T-2 exposure compared with the control group. Its activity was also more increaser in all the PRO exposure than the control group. Nonetheless, an enormous decline was detected in all the MTP exposures compared with the corresponding T-2 exposures. Moreover, its activity also declined in the low-dose MTP exposure compared with the corresponding PRO exposure (Fig. 2D).

VTG content

The content of VTG was apparently reduced in all individual exposures (except for the middle-dose T-2 and PRO exposures) compared with the control group. An apparent reduction was also monitored in the high-dose MTP exposure compared with the control group. Nonetheless, its content was apparently induced in the high-dose MTP exposure compared with the corresponding T-2 and PRO exposures (Fig. 3).

Gene expression analysis

Expressions of genes related to oxidative stress

The expression of *Mn-sod* was remarkably diminished in the high-dose T-2 exposure and the middle-dose MTP exposure compared with the control group. A diminution was also seen in the low- and middle-dose MTP exposures compared with the corresponding individual exposures. However, its expression was augmented in the high-dose MTP exposure compared with the corresponding T-2 exposure. Significant increase was also noticed in the low-dose PRO exposure compared to the control (Fig. 4A). The expression of *cat* was stimulated in the low-dose T-2 and PRO exposures compared with the control group. However, its expression was dramatically decreased in the high-dose PRO exposure and the middle-dose MTP exposure compared with the control group. A dramatic inhibition was also observed in the

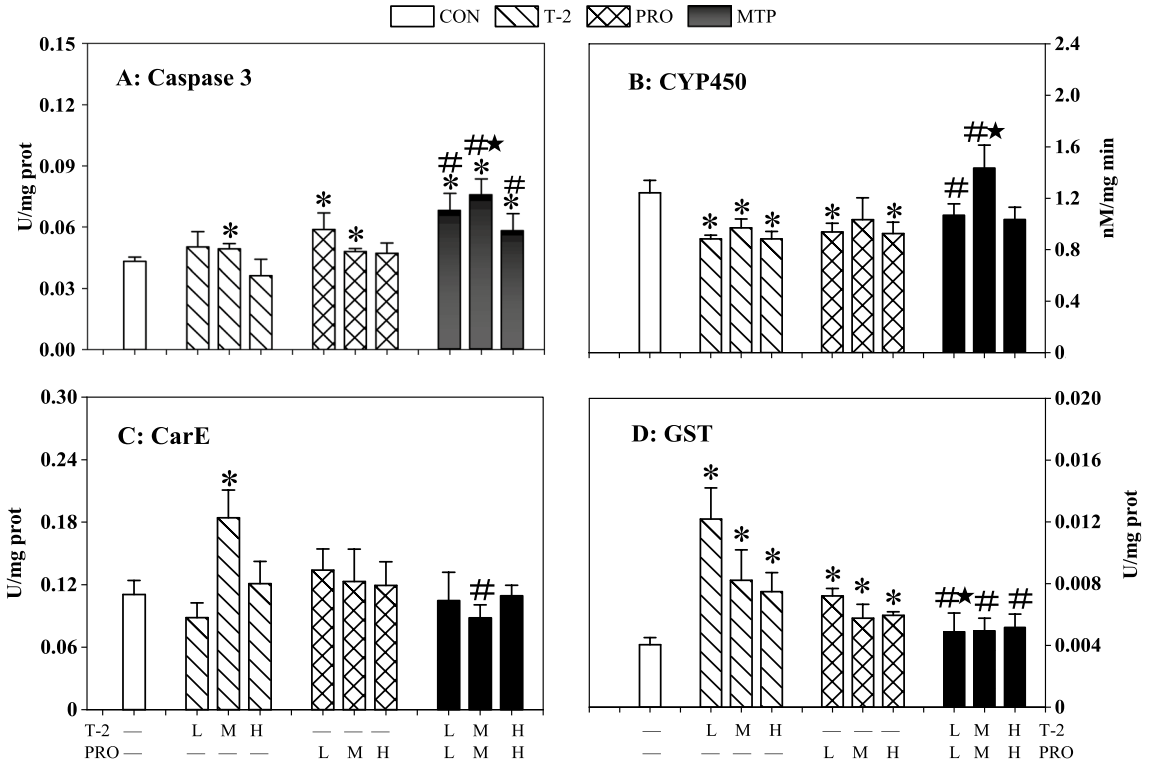

Fig. 2 The activities of apoptotic and detoxifying enzymes in zebrafish treated with T-2 PRO and their combinations. **A** Caspase 3, **B** CYP450, **C** CarE, D GST. Each bar represents the mean ± standard deviation (n = three replicates consisting of a group of 250 fish). * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding T-2 exposure; ★$p < 0.05$ significant difference relative to the corresponding PRO exposure. *CON* control, *T-2* T-2 toxin, *PRO* propiconazole, *MTP* the mixture of T-2 and PRO. *L* low concentration, *M* middle concentration, *H* high concentration

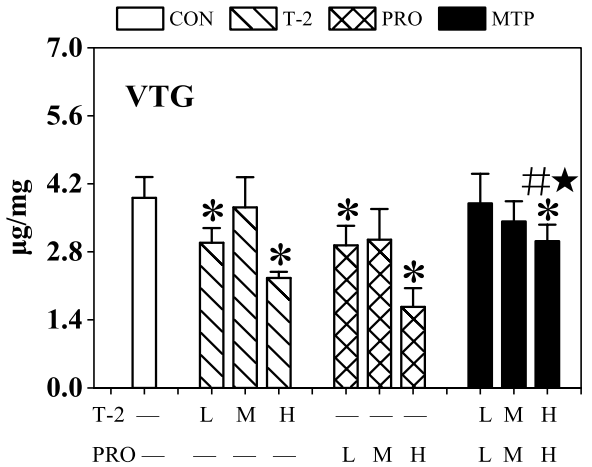

Fig. 3 The VTG level of zebrafish treated with T-2 PRO and their combinations. Each bar represents the mean ± standard deviation (n = three replicates consisting of a group of 250 fish). * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding T-2 exposure; ★$p < 0.05$ significant difference relative to the corresponding PRO exposure. *CON* control, *T-2* T-2 toxin, *PRO* propiconazole, *MTP* the mixture of T-2 and PRO. *L* low concentration, *M* middle concentration, *H* high concentration

middle-dose MTP exposure compared with the corresponding individual exposures (Fig. 4B).

Expressions of genes associated with cellular apoptosis and immune system

Figure 5 summarizes the changes in the expressions of genes involved in cellular apoptosis and the immune system. The expression of *cas9* was observably diminished in the middle-dose PRO and MTP exposures compared with the control group. Concurrently, a noticeable decline was also seen in the low-dose MTP exposure compared with the corresponding PRO exposure. Conversely, its expression was elevated in the high-dose MTP exposure compared with the corresponding T-2 exposure. The expression of cas9 was also significantly elevated in the low-dose PRO exposure compared with the control group (Fig. 5A). The expression of *p53* was notably elevated in the middle- and high-dose T-2 exposures compared with the control group. However, its expression was notably reduced in the middle- and high-dose MTP exposures compared with the corresponding T-2 exposures (Fig. 5B). The expression of *bax* was notably raised in the low-dose PRO exposure compared with the

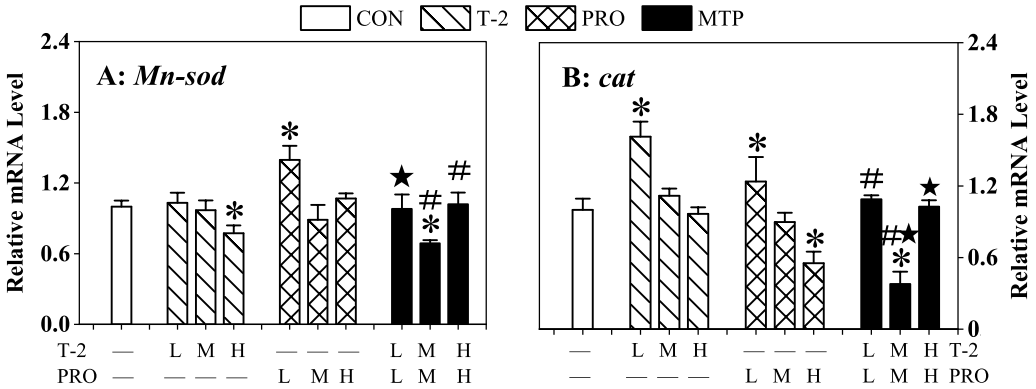

Fig. 4 Influences on expressions of genes associated with the anti-oxidative systems in fish administrated with T-2 PRO and their combinations. **A** *Mn-sod*, **B** *cat*. Each bar represents the mean ± standard deviation (n = three replicates consisting of a group of 50 fish). * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding T-2 exposure; ★$p < 0.05$ significant difference relative to the corresponding PRO exposure. *CON* control, *T-2*T-2 toxin, *PRO* propiconazole, *MTP* the mixture of T-2 and PRO. *L* low concentration, *M* middle concentration, *H* high concentration

control group. In contrast, its expression was notably reduced in the low-dose MTP exposure compared with the corresponding PRO exposure (Fig. 5C).

The expression of *cas3* was diminished in the high-dose of T-2 exposure and the middle-dose of PRO and MTP exposure compared with the control group. Its expression was diminished in the middle-dose PRO and MTP exposures compared with the corresponding T-2 exposure. A diminution was also noted in all the MTP exposures (except for the high-dose MTP exposure) compared with the corresponding PRO and T-2 exposures. By contrast, its expression was markedly increased in the low-dose PRO exposure compared with the control group (Fig. 5D). The expression of *bcl-2* was substantially reduced in the middle-dose PRO and MTP exposures compared with the control group. A substantial reduction was monitored in the high-dose T-2 exposure compared with the control group. However, its expression was substantially elevated in the low- and high-dose MTP exposures compared with the corresponding T-2 exposures (Fig. 5E). The expression of *IL-8* was pronouncedly induced in the middle- and high-dose PRO exposures compared with the control group. In contrast, a pronounced down-regulation was monitored in the middle- and high-dose MTP exposures compared with the corresponding PRO exposures (Fig. 5F).

Expressions of genes involved in the endocrine system

The expression of *TRα* was promoted in the low-dose PRO and MTP exposures compared with the control group. A significant increase was also monitored in the low-dose MTP exposure compared with the corresponding T-2 exposure. Nonetheless, its expression was decreased in the high-dose MTP exposure compared with the corresponding PRO exposure (Fig. 6A). The expression of *TRβ* was pronouncedly up-regulated in all exposures (except for the high-dose MTP exposure) compared with the control group. On the contrary, a pronounceable inhibition was discovered in the middle- and high-dose MTP exposures compared with the corresponding PRO exposures (Fig. 6B). The expression of *tsh* was prominently improved in all exposures (except for the high-dose MTP exposure) compared with the control group. However, a steep inhibition was observed in the high-dose MTP exposure compared with the corresponding T-2 and PRO exposures. Its expression was also steeply inhibited in the middle-dose MTP exposure compared with the corresponding PRO exposure (Fig. 6C).

The expression of *crh* was appreciably enhanced in all exposures compared with the control group. An appreciable enhancement was also noted in the high-dose MTP exposure compared with the corresponding T-2 exposure. In contrast, its expression was appreciably weakened in the high-dose MTP exposure compared with the corresponding T-2 exposure. It was worth noting that the expression of *crh* increased with the increase of concentration in the T-2 treatment, but its expression was negatively correlated with the dose of PRO and MTP treatment (Fig. 6D). The expression of *cyp19a* was surprisingly elevated in the low-dose T-2 exposure compared with the control group. In contrast, its expression was surprisingly diminished in the low- and high-dose MTP exposures compared with the control group, as well as the corresponding T-2 and PRO exposures (Fig. 6E). The expression of *vtg1* was significantly induced in the individual and MTP exposures (except for the middle-dose T-2 exposure and the high-dose MTP exposure) compared with the control group. An induction was also

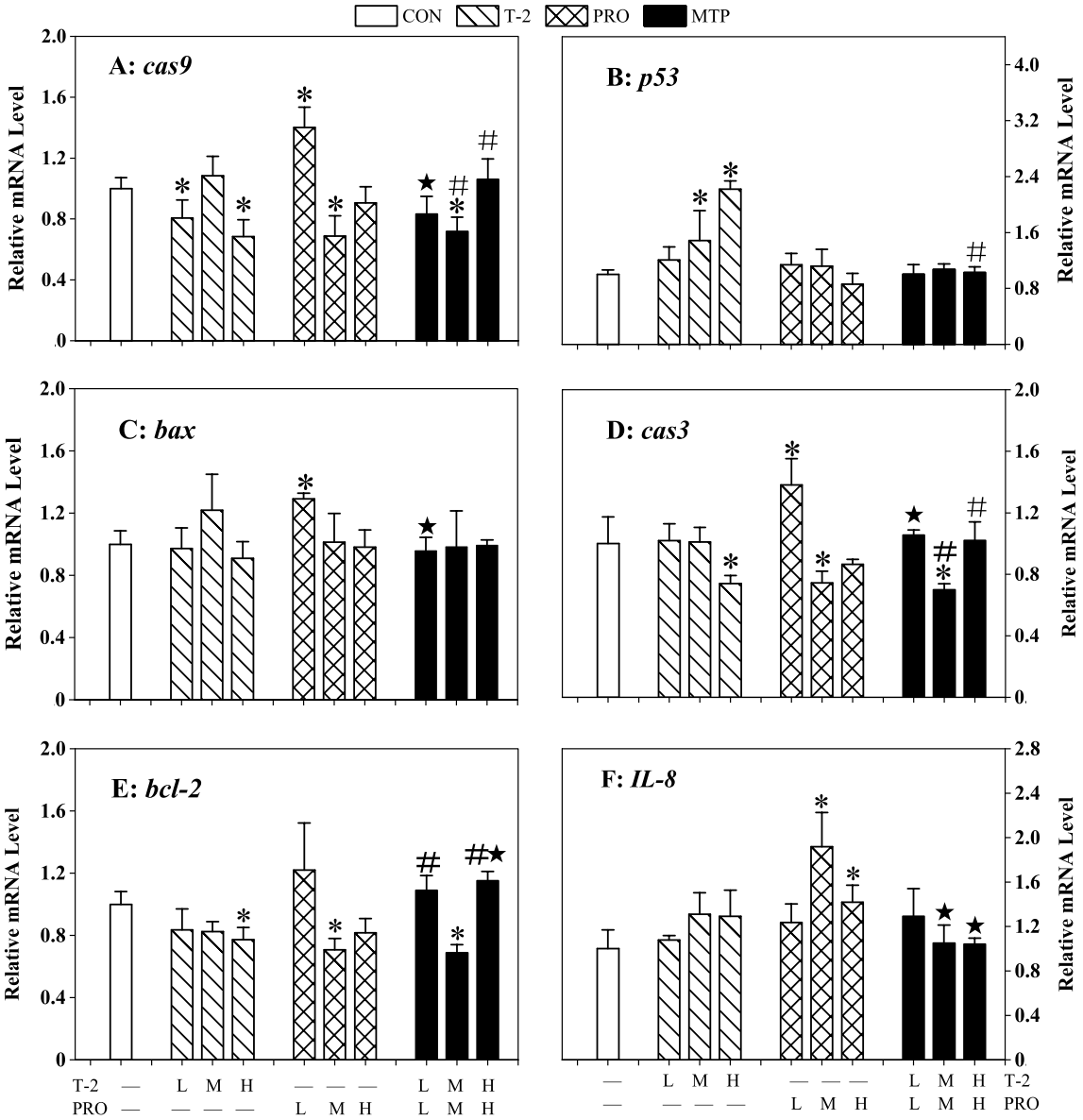

Fig. 5 Influences upon expressions of genes involved in the apoptosis and immune-related of fish administrated with T-2 PRO and their combinations. **A** *cas9*, **B** *p53*, **C** *bax*, **D** *cas3*, **E** *bcl-2*, **F** *IL-8*. Each bar represents the mean ± standard deviation (n = three replicates consisting of a group of 50 fish). * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding T-2 exposure; ★$p < 0.05$ significant difference relative to the corresponding PRO exposure. *CON* control, *T-2* T-2 toxin, *PRO* propiconazole; *MTP* the mixture of T-2 and PRO. *L* low concentration, *M* middle concentration, *H* high concentration

noticed in the low- and middle-dose MTP exposures compared with the corresponding T-2 exposures. On the contrary, its expression was markedly inhibited in the high-dose MTP exposure compared with the corresponding PRO exposure (Fig. 6F).

Throughout mRNA expression variations

A heatmap analysis consisting of 16 genes was established upon the exposures to T-2, PRO, and their mixtures for exploring the overall changes in gene expressions. The results showed that the distance between the experimental groups was far apart. T-2 and PRO individual exposure groups were closed together, followed by single exposure group and combined exposure group, which can be clustered together. The control group was far away from most treatment groups. It was shown that gene expression in each treatment group was specific compared to the control group. At the gene clustering level,

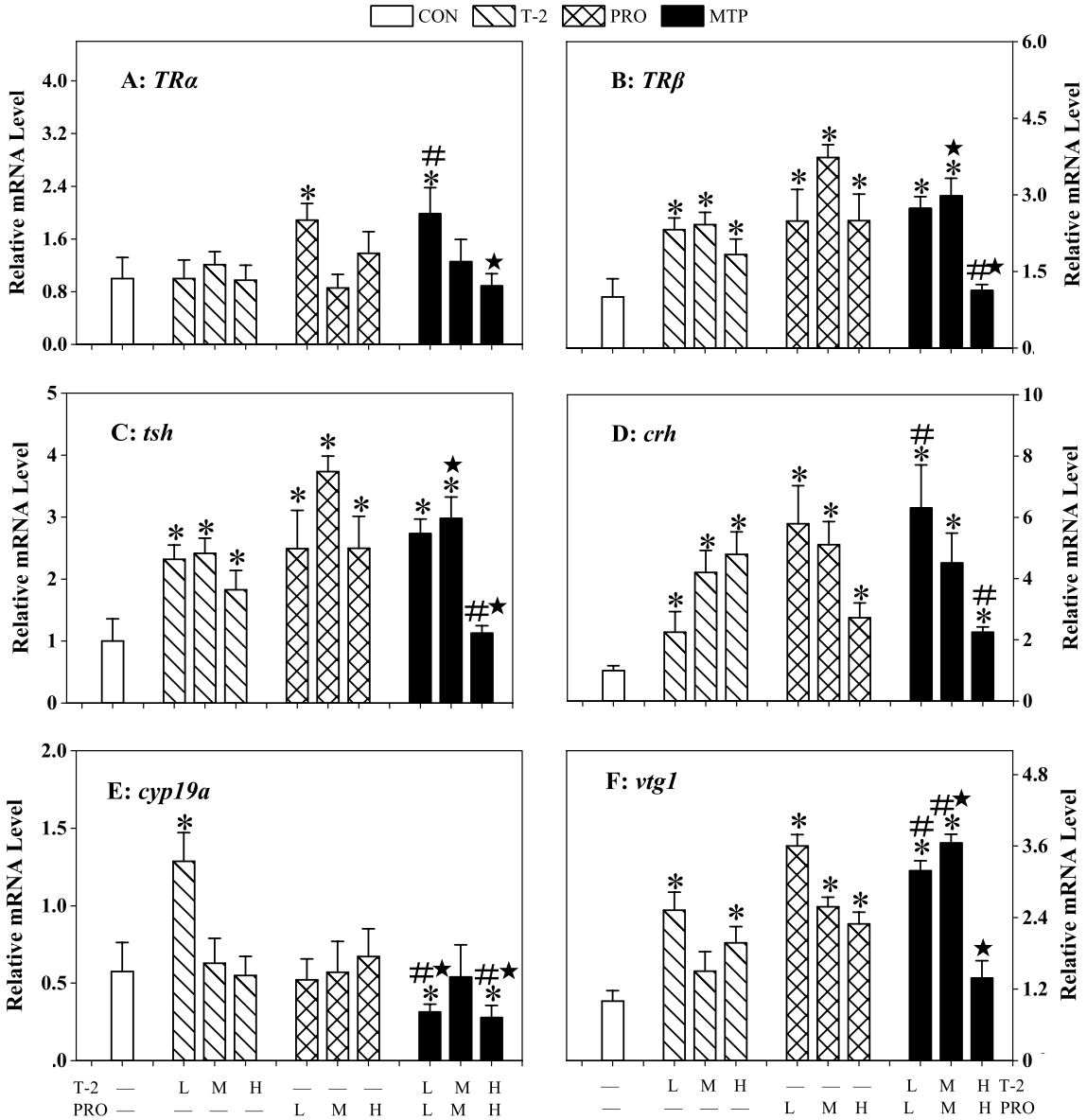

Fig. 6 Influences upon expressions of genes involved in the endocrine system in zebrafish treated with T-2 PRO and their combinations. **A** *TRα*, **B** *TRβ*, **C** *tsh*, **D** *crh*, **E** *cyp19a*, **F** *vtg1*. Each bar represents the mean ± standard deviation (n = three replicates consisting of a group of 50 fish). * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding T-2 exposure; ★$p < 0.05$ significant difference relative to the corresponding PRO exposure. *CON* control, *T-2* T-2 toxin, *PRO* propiconazole, *MTP* the mixture of T-2 and PRO. *L* low concentration, *M* middle concentration, *H* high concentration

the gene expression of *bax* and *TRα* was closed together, meaning that gene expression was similar. In addition, the expression of *tsh* and *vtg1* genes was an up-regulated trend, with consistent expression changes (Fig. 7).

Discussion

Acute toxicity test on zebrafish provides a fundamental understanding of the primary effects of chemicals [51]. Our results demonstrated that T-2 had stronger acute toxicity to zebrafish compared with PRO. A previous study has demonstrated that the 96-h LC_{50} value of PRO to zebrafish juveniles is 8.25 mg a.i. L^{-1} [52], which is not consistent with our data (the 96-h LC_{50} value of PRO to zebrafish larvae was 17.16 mg a.i. L^{-1}). This slight difference might likely be attributed to the different life stages of zebrafish determined [53]. Another investigation has demonstrated that T-2 has developmental toxicity in zebrafish [54]. In the present study, the synergistic effects

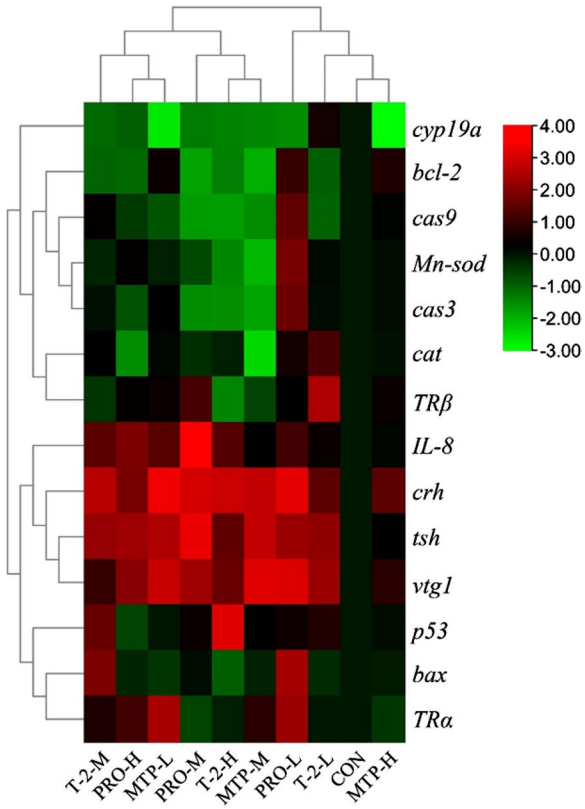

Fig. 7 Heat map evaluation for analyzing throughout variations of mRNA expression. *CON* control, *T-2* T-2 toxin, *PRO* propiconazole, *MTP* the mixture of T-2 and PRO, *L* low concentration, *M* middle concentration, *H* high concentration

of T-2 and PRO suggested that there might be a potential risk in the co-existence of these chemicals in agricultural products. Consequently, it is urgently necessary to elucidate the combined toxic mechanism of T-2 and PRO to avoid underestimating the combined toxicity.

There are oxidation and antioxidant regulation mechanisms in organisms [55]. When exogenous pollutants stimulate the organism, the levels of antioxidant defense factors change, and the organism enters a state of oxidative stress. CAT and SOD are critical antioxidant enzymes [56]. The present study found that the SOD activity was enhanced when exposed to low doses of T-2 and PRO. This transient increase suggests that zebrafish embryos responded to the concentration of T-2 and PRO in the form of antioxidant, which may be a mechanism of self-protection [57]. However, the SOD activity in zebrafish was dramatically inhibited by MTP exposure compared with individual chemicals, suggesting that MTP exposure increased oxidative stress in zebrafish. MDA is the main by-product of lipid peroxidation, and its level may reflect the status of lipid peroxidation in organisms [58]. The content of MDA was significantly induced in the middle-dose MTP exposure, suggesting that combined exposure resulted in lipid peroxidation. Exposure to low-dose T-2 significantly increased the CAT content compared with the control group, which might be attributed to the fact the induced CAT activity abolished oxidative damage. Based on the results above, we deduced that T-2, PRO, and MTP exposures enhanced the ROS production in zebrafish embryos and activated antioxidant defense afterward. As suggested by Li et al. T-2 induces apoptosis through ROS-mediated mitochondrial pathway in poultry [59]. Exposure to PRO induced mouse liver oxidative stress [60]. In contrast, antioxidant responses could not completely eliminate the excess ROS in MTP exposures, rendering oxidative impairment.

In response to exogenous toxic substances, ROS production and apoptosis are closely related in zebrafish [61]. Apoptosis affects the growth and development of organisms, as well as different systemic mechanisms of toxicity. Caspase 3 is an important executioner of apoptosis and is activated to cleaved-caspase 3 in an apoptotic state [62]. In this study, we demonstrated that the caspase 3 activity was notably induced after exposure to MTP compared with the control group, implying that T-2 and PRO exerted a toxic effect on apoptosis in zebrafish embryos. The expression of *p53* was considerably enhanced in the medium- and high-dose T-2 exposures compared with the control group, revealing that the apoptotic effect caused by T-2 was enhanced. Our data supported the previous finding of Xian et al. [63] that exposure to T-2 induces apoptosis in the human renal tubular epithelial cells HK-2. An increased amount of ROS can cause apoptosis during embryogenesis [64]. The continuation of apoptosis signaling leads to lipid peroxidation and cellular death [65]. Oxidative stress mediates apoptosis through mitochondria, death receptors (TN-FR1, Fas, TRAILR2, DR3, DR4, DR5), and endoplasmic reticulum stress and may also induce apoptosis by activating the mitogen-activated protein kinase pathway, nuclear transcription factor κB, and caspase [66, 67]. CYP450, CarE, and GST are the primary detoxification enzymes in organisms [68]. CarE catalyzes carboxylate hydrolysis in phase-I biotransformation to produce alcohols and acids. The CarE activity was appreciably increased in the middle-dose T-2 exposure, implying that exposure to the middle-dose T-2 enhanced the detoxification in embryos. GST is a superfamily of enzymes that catalyze detoxification reactions, commonly used as a marker of detoxification in vertebrate and invertebrate studies [69, 70]. The CarE and GST activities were significantly inhibited by simultaneous exposure to T-2 and PRO, suggesting that the detoxification system of zebrafish embryos was compromised when exposed to MTP. We found that exposure to the middle-dose MTP increased the activity of

CYP450, which might boost the active metabolism of T-2 and PRO in zebrafish.

VTG is a biomarker of environmental estrogen exposure and provides a source of nutrition for fish embryos at the early stages of larval development [71]. Our results found that the level of VTG was appreciably lower in the low- and high-dose individual chemical exposures compared with the control group. However, it was significantly increased in the high-dose MTP exposure, indicating that T-2 and PRO could greatly affect estrogen levels in zebrafish. T-2 has exhibited a concentration-dependent inhibition effect on the cell reproductive system [72]. Besides, the sex hormone is regulated by the hypothalamic–pituitary–gonadal (HPG) axis. Consequently, our results showed that the zebrafish endocrine system was affected by T-2 and PRO, may leading to disrupted fish reproduction. Apoptosis is continuously induced when cells are subjected to oxidative stress or DNA damage [73]. The expressions of apoptosis-related genes such as *p53, bax, cas3,* and *cas9,* were inhibited. In addition, the expressions of *cas3* and *cas9* were more strongly inhibited in the middle-dose MTP exposure. Additionally, oxidative stress can also affect immune function. The expression of *IL-8* was conspicuously decreased in the MTP exposure, indicating that the immune system was compromised when zebrafish were exposed to MTP. T-2 has been shown to have immunomodulatory functions, and in this study, the expression of *IL-8* tended to increase in the medium and high concentration T-2 treatments [74]. Its expression was significantly increased in the middle -dose of PRO, indicating that the PRO could lead to immunity boosting. However, with the further increase of PRO concentration, the immune promoting effect tended to decrease, which means that the immune effect in the high concentration of PRO on zebrafish was hindered. Endocrine disruptors interact with endocrine properties in animals by either mimicking or blocking, which can affect organismal immunity, growth, metabolism, and reproduction [75]. Accumulating evidence has summarized that food intake is the primary source of exposure in humans, which can lead to female infertility, precocious puberty, and other related diseases, while such exposure can reduce sperm and sexual functions for men [76, 77]. The six genes associated with endocrine disruption we analyzed were *TRα, TRβ, tsh, crh, cyp19a,* and *vtg1*. The HPG is a crucial regulator of reproduction in mammals [78]. Under normal conditions without exposure to external pollutants, the level of *vtg* in fish larvae is low. When fish larvae are exposed to exogenous environmental pollutants, *vtg* is synthesized in the liver of fish larvae. Therefore, the gene of *vtg* has been used as a sensitive molecular biological biomarker for detecting the endocrine-disrupting effects of estrogen. The expression of *vtg1* was appreciably increased in all the T-2 and PRO exposures (except for the medium-dose T-2 exposure) compared with the control group, implying that both T-2 and PRO exerted estrogenic disrupting capacity on zebrafish embryos [79, 80]. The expression of *vtg1* was also greatly up-regulated in the low- and medium-dose MTP exposures compared with the corresponding single exposures, confirming that T-2 and PRO had an enhancing effect on the estrogen of zebrafish. The gene expression in response to chemical exposure represents the interaction sites of drugs and zebrafish [81]. *cyp19a* is mainly expressed in the sex gland. It encodes aromatase that alters the ratio of estrogens to androgens in fish, affecting the sex of the fish [82]. Its expression was tremendously down-regulated in the MTP exposure compared with the corresponding T-2 and PRO exposures, indicating that MTP exposure probably affected the reproduction of *D. rerio*.

The hypothalamic–pituitary–thyroid (HPT) axis regulates fish growth and development [83]. In the HPT axis, corticotropin-releasing *crh* secreted by the hypothalamus induces the pituitary gland to secrete *tsh* [84]. The *tsh* is an essential gene for the secretion of thyroid hormones. Genes of *TRα* and *TRβ* are receptors for thyroid hormones [85]. In the present study, the expressions of *crh* and *tsh* were conspicuously induced in the T-2 and PRO exposures compared with the control group, indicating that both T-2 and PRO had an inductive effect on the hypothalamus and pituitary gland of zebrafish. However, the expressions of *tsh* and *crh* were significantly suppressed when exposed to high-dose MTP compared with the T-2 exposure, implying that a high concentration of T-2 and PRO had negative feedback on the HPT axis. In addition, the expressions of *TRα* and *TRβ* were also remarkably altered by the MTP exposures. The varying degrees of inhibition in the high-dose MTP exposure implied that T-2 and PRO had potential disruptive effects on the thyroid gland of zebrafish. Similar to the obtained results, PRO and T-2 have been reported that induced thyroid and metabolism disruption [86, 87]. Inhibition of thyroid axis-related genes might weaken the synthesis and secretion of thyroid hormones, thereby inhibiting the development of fish larvae.

Investigation of mixed toxicity of T-2 and PRO at multiple endpoints contributes to a comprehensive understanding of the effects of mycotoxin and pesticide mixtures on zebrafish [88, 89]. Enzymatic activities of SOD, GST, and caspases 3 were obviously impacted when exposed to T-2 PRO or their mixture. Moreover, genes associated with the endocrine system such as *tsh, crh,* and *vtg1,* were significantly changed after exposure to MTP. When an organism is exposed to chemical contaminants, changes in enzyme activity can reflect the degree of cell

damage, and gene expression can provide changes at the mRNA level [90]. Therefore, we deduced that the mixture of T-2 and PRO might disturb the growth, development, and reproduction of human beings. However, the mixture mechanism of T-2 and PRO at the transcription and metabolomics levels needs to be further explored.

Conclusions

T-2 exhibited higher acute toxicity to zebrafish embryos than PRO. The combination of T-2 and PRO had an acute synergistic effect on zebrafish. Most individual and combined exposures significantly changed the SOD and GST activities compared with the control group. Furthermore, the expressions of HPT and HPG axis-related genes (*tsh, crh, TRβ, cyp19a*, and *vtg1*) immune system-related genes (*bcl-2* and *IL-8*), and cellular apoptosis-related genes (*cas3* and *cas9*) were tremendously disturbed in the combined exposure group compared with individual exposures. These changes of indexes determined might provide early warning on the mixture effects of mycotoxin and pesticides. Collectively, the impacts of chemical synergy on the toxicity of combined contaminants should be considered prior to assessing the risk in agriculture products.

Abbreviations

T-2	T-2 toxin
PRO	Propiconazole
MTP	The mixture of T-2 toxin and propiconazole
LC_{50}	Median lethal concentration
AI	Additive index
L	Low concentration
M	Middle concentration
H	High concentration
SOD	Superoxide dismutase
CAT	Catalase
MDA	Malondialdehyde
CYP450	Cytochrome P450
CarE	Carboxylesterase
GST	Glutathione-S-transferase
VTG	Vitellogenin
HPT	Hypothalamic–pituitary–thyroidal
HPG	Hypothalamic–pituitary–gonadal

Supplementary Information

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Additional file 1: **Table S1**. Gene primer sequences in real-time quantitative PCR reaction. **Table S2**. Lethal toxicity of T-2 toxin and propiconazole to zebrafish, separately and combined.

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Author contributions

SZ: conceptualization, data curation, formal analysis, writing—original draft, writing–review and editing. XL: data curation, formal analysis, writing–original draft, project administration. LL: conceptualization, data curation, formal analysis, writing–original draft, project administration. CL: conceptualization, investigation, methodology, resources. TL: conceptualization, data curation, formal analysis. HZ: investigation, methodology, resources. JZ: supervision. YW: conceptualization, writing—original draft, supervision, funding acquisition, project administration, writing—review and editing. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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