Exposure to trace levels of metals and fluoroquinolones increases inflammation and tumorigenesis risk of zebrafish embryos

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Exposure to trace-level heavy metals and antibiotics may elicit metabolic disorder, alter protein expression, and then induce pathological changes in zebrafish embryos, despite negligible physiological and developmental toxicity. This study investigated the single and combined developmental toxicity of fluoroquinolones (enrofloxacin [ENR] and ciprofloxacin [CIP]) (≤0.5 μM) and heavy metals (Cu and Cd) (≤0.5 μM) to zebrafish embryos, and molecular responses of zebrafish larvae upon exposure to the single pollutant (0.2 μM) or a binary metal-fluoroquinolone mixture (0.2 μM). In all single and mixture exposure groups, no developmental toxicity was observed, but oxidative stress, inflammation, and lipid depletion were found in zebrafish embryos, which was more severe in the mixture exposure groups than in the single exposure groups, probably due to increased metal bioaccumulation in the presence of ENR or CIP. Metabolomics analysis revealed the up-regulation of amino acids and down-regulation of fatty acids, corresponding to an active response to oxidative stress and the occurrence of inflammation. The up-regulation of antioxidant and immune proteins revealed by proteomics analysis further confirmed the occurrence of oxidative stress and inflammation. Furthermore, the KEGG pathway enrichment analysis showed a significant disturbance of pathways related to immunity and tumor, indicating the potential risk of tumorigenesis in zebrafish larvae. The findings provide molecular-level insights into the adverse effects of heavy metals and antibiotics (especially in chemical mixtures) on zebrafish embryos, and highlight the potential ecotoxicological risks of trace-level heavy metals and antibiotics in the environment.

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1. Introduction

Fluoroquinolones (FQs) such as enrofloxacin (ENR) and ciprofloxacin (CIP) are mainly administered to food animals (e.g., fish, pigs, and chicken) for the prevention and treatment of bacterial infections [1,2]. Because of their large uses worldwide, CIP and ENR were detected in environmental water samples at concentrations from several nM to several tens of μM [3–5]. For example, the ENR concentrations range from 0.016 to 0.068 nM in surface waters [3], are up to hundreds of nM in treated wastewater effluents from hospitals and livestock farms [4,6], and even as high as 2.50 μM in effluents of pharmaceutical wastewater [7]. More alarmingly, the CIP concentration was up to 93.6 μM, 2.72 μM, 0.794 μM, and 1.04 nM in the effluents of pharmaceutical wastewater, hospitals, livestock farms, and surface waters, respectively [3,4,6,7]. Thus, it is important to study the toxicity of ENR and CIP to aquatic organisms.

Studies have reported that ENR and CIP could induce growth inhibition [8.63 μM ENR and 90.5 μM CIP], reproduction inhibition...
(17.5 μM ENR and 90.5 μM CIP), oxidative stress (0.278 μM ENR and 0.302 μM CIP), lipid metabolic disturbance (0.003 μM ENR and CIP), and cardiotoxicity (4246 μM CIP) to non-target aquatic organisms [8–10]. Antibiotic exposure at environmentally-relevant levels may not inhibit the development, growth, and reproduction of aquatic organisms, but instead cause oxidative stress, inflammation, and metabolic disturbance [11–14]. Indeed, the exposure of zebrafish embryos to 0.028 and 1.39 μM ENR for 3 days increased the catalytic activity of glutathione peroxidase but decreased the catalytic activity of glutathione-S-transferase [14]. Similarly, the exposure of zebrafish embryos to sulfamethoxazole (0.395 μM) increased the total antioxidant capacity, the gene expression of key proinflammatory cytokines, and the production of corresponding proteins in zebrafish embryos [13]. The exposure of Nile tilapia to 0.001 μM oxytetracycline and 0.001 μM sulfamethoxazole increased the antioxidase activity, suppressed innate immunity, and stimulated inflammatory and detoxification responses in fish [12]. Nonetheless, previous studies mainly focused on the exposure to the individual antibiotic or the mixtures of antibiotics [9] and rarely studied the toxicity of the mixtures of antibiotics and heavy metals to aquatic organisms.

Assessing the toxicity of chemical mixtures to aquatic organisms is challenging due to their potential synergistic, additive, or antagonistic effects [15]. For example, the co-exposure to 5 μM 1-H-benzotriazole and 1 μM Cd decreased the acute toxicity of Cd and alleviated the Cd-induced liver atrophy in zebrafish [16]. However, no-effect-concentrations of dibenzothiophene (1 μM) and Cd (0.2 μM) in mixtures caused morphological defects of thyroid and developmental toxicity in zebrafish embryos [17]. Despite these progresses, the studies on the joint toxicity of FQs and heavy metals at environmentally relevant concentrations to aquatic organisms are still limited, which is imperative because FQs and heavy metals often coexisted in natural waters [18,19].

Therefore, this study investigated the development and metabolic and proteomic responses of zebrafish embryos upon exposure to individual FQs (ENR or CIP), heavy metals (Cu or Cd), or binary mixtures of one fluorquinolone and one heavy metal. The changes of reactive oxygen species (ROS), macrophage, and lipids in zebrafish larvae were examined using in vivo fluorescence imaging and in vitro fluorescence quantification. The inflammation and tumorigenesis potential of zebrafish embryos were further explored with metabolomics and proteomics analyses. This study provides novel insights into the potential risks of antibiotics and heavy metals to aquatic organisms.

2. Materials and methods

2.1. Zebrafish and chemicals

Adult zebrafish (Tübingen line, 3-month-old, Danio rerio) was supplied by Nanjing EzErinka Biotech Company. CIP (purity >98.0%), Cd(NO3)2·H2O (purity >99.0%), and 2-7′-dichlorodihydroxy-drofluorescein diacetate (H2DCFDA) were purchased from Macklin Biochemical Co., Ltd (China), ENR (purity >98.0%) from Aladdin Industrial Corporation, CuSO4·5H2O (purity >99.0%) from Sangon Biotech (China) Co., Ltd, 1-phenyl-2-thiourea (PTU) from Yuanye Bio-Technology Co., Ltd (China), neutral red and Nile red from Eon Chemical Technology Co., Ltd (China), and tricine from Tixiai Co., Ltd (China).

2.2. Zebrafish husbandry, egg collection, and exposure tests

Adult zebrafish were maintained in an automatic circulation system at 28 °C and a 14 h/10 h light/dark cycle. Details on zebrafish husbandry and egg collection are given in Text S1. The developmental toxicity tests were conducted in 12-well plates with 10 embryos per well in a constant temperature incubator at 28 °C according to OECD236 [20]. The embryos were exposed to the single pollutant or mixtures from 12 to 96 h post-fertilization (hpf). The exposure tests were conducted in triplicate (i.e., a total of 30 embryos for each treatment). The spontaneous embryo movement was counted by an inverted microscope (JSM6, Olympus, Japan) in 1-min intervals at 24 hpf. The heart rate was determined by an inverted microscope (BX51, Olympus, Japan) via counting heartbeats of embryos in 30-s intervals at 48 hpf. The mortality rate and hatching rate at 96 hpf were recorded.

To investigate the developmental toxicity of metals and antibiotics to zebrafish embryos, the stock solution of Cu (50 mM), Cd (50 mM), CIP (50 μM), or ENR (50 μM) was added to the E3 medium (2 mM CaCl2, 0.5 mM MgSO4, 0.75 mM NaHCO3 and 0.08 mM KCl, pH 7.0 ± 0.5, and electrical conductivity 289 ± 5 µS cm−1) according to OECD236 [20] to reach the metal or antibiotic concentration range of 0.0005–0.5 μM. For the toxicity tests of metal-antibiotic mixtures, the concentration of Cu or Cd was fixed at 0.2 μM, and the concentration ratio of metal to antibiotic was set as 0, 0.25, 0.5, 1, and 2. Control groups were free of Cu, Cd, CIP, or ENR. Detailed exposure conditions are shown in Table S1. The method for detecting the interactions between ENR/CIP and Cu/Cd is described in Text S2.

2.3. In vivo fluorescence imaging and quantification of ROS, macrophage, and lipid

During the exposure tests, 200 μM PTU was added into the exposure solution to inhibit pigmentation at 24 hpf. The 96 hpf embryos exposed to 0.2 μM of metal or/and 0.2 μM of antibiotic were immersed in 25 μM of H2DCFDA for 40 min. 2.5 μg mL−1 of neutral red for 25 min, and 0.5 μg mL−1 of Nile red for 30 min at 28 °C in the dark to determine the ROS production, the macrophages accumulation, and lipid content, respectively. After incubation, the embryos were rinsed with culture media and phosphate-buffered solution, and then were anesthetized with 0.01% tricaine. The stained embryos (3 embryos for each group) were observed at excitation/emission wavelengths of 485/530 nm, 450/610 nm, and 530/635 nm, respectively, using an inverted fluorescence microscope (Nikon Eclipse Ti, Japan).

Another fraction of the incubated embryos was homogenized by sonicating for 5 min (25 kHz). The suspensions were centrifuged at 5000 rpm for 10 min, and then the supernatants were collected. The contents of ROS, macrophage, and lipid were determined by measuring the fluorescence of the supernatants by a fluorescence spectrometer (Cary Eclipse, Varian, USA) at excitation/emission of 485/530 nm, 450/610 nm, and 530/635 nm, respectively.

2.4. Determination of Cu and Cd concentrations in exposure solutions and embryos

The embryos were exposed to single Cu or Cd (0–0.5 μM) and metal-antibiotic mixtures in a 120-mm glass Petri dish with 100 embryos from 12 hpf to 96 hpf, the concentration of Cu or Cd in mixtures was fixed at 0.2 μM, and the concentration ratio of metal to antibiotic was set as 0, 0.25, 0.5, 1, and 2. Detailed exposure conditions are shown in Table S1. Three replicates were independently performed for each treatment. Exposure solutions were replaced every day, and 10 mL exposure solutions prior to and after daily exposure were collected to quantify the concentration of metals in the solution before and after exposure. After exposure, the 96 hpf zebrafish embryos from the control and treatment groups were washed three times with ultrapure water, and the embryos were collected in a 10-mL centrifuge tube with 5 mL
ultrapure water. The embryo suspensions were sonicated for 5 min (25 kHz), and then digested using 5 mL HNO₃ at 150 °C for 45 min on an electric heating board. After digestion, the residue was dissolved in 5 mL 2% HNO₃. Cu and Cd concentrations in pre- and post-exposure solutions and digested samples were quantified by inductively coupled plasma mass spectrometry (ICP-MS, X Series II, Thermofisher, USA).

2.5. Metabolomics analysis

The 96 hpf embryos exposed to metals (0.2 μM), antibiotics (0.2 μM), metal (0.2 μM)-antibiotic (0.2 μM) mixtures were collected for metabolomics analysis, and the non-exposed 96 hpf embryos were used as the control. Zebrafish embryos were washed with ultrapure water, and a total of 350 (7 × 50) embryos were collected for each exposure group (i.e., 50 embryos in a 1.5 mL centrifuge tube per replicate, and 7 replicates per treatment). Then, the metabolite extraction was performed. More detailed information on sample preparation and metabolomic analysis is described in Text S3, following our previous study [21]. The differentially expressed metabolites (DEMs) (variable importance in projection [VIP] value of orthogonal partial least squares discriminant analysis [OPLS-DA] > 1 and the p value [Student’s t-test] < 0.05) were selected.

2.6. Proteomics analysis

The 96 hpf embryos exposed to metals (0.2 μM), antibiotics (0.2 μM), and metal (0.2 μM)-antibiotic (0.2 μM) mixtures were collected for proteomics analysis, with the non-exposure embryos as the control. For each treatment, 350 embryos were pooled as one sample and washed with ultrapure water, and the protein extraction and trypsin digestion were then performed. Differentially expressed proteins (DEPs) (fold change >1.50 or < 0.67) were selected. The DEPs data were processed using Gene Ontology (GO) annotations, Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation, and subcellular localization. More detailed information on sample preparation and proteomic analysis is described in Text S4 [22].

2.7. Statistical analysis

SPSS 18.0 (PASW Statistics, IBM Company) was used for the statistical analysis. The data were presented as mean ± standard deviation (SD). One-way ANOVA was performed to compare the means of different exposure groups at p < 0.05. OPLS-DA was performed using SIMCA (V16.0.2) software. The Venn plots of DEPs were generated in Gephi 0.9.2. KEGG pathway classification of proteomics was performed according to the instruction of the database (http://www.string-db.org/). The protein-protein interaction (PPI) was drawn online using the STRING protein interaction network database (https://www.string-db.org/). Figures were plotted using Origin 2020b.

3. Results and discussion

3.1. Developmental toxicity of metals or/and FQs

In the single exposure groups (Fig. 1), environmental concentrations of Cu, Cd, ENR, or CIP had no significant effects on the death rate, hatching rate, spontaneous movement, and heart rate of zebrafish embryos (p > 0.05). Similar to the present study, exposure to 0.134 μM clarithromycin, 0.279 μM florfenicol, or 0.359 μM sulfamethazine did not induce the developmental inhibition of embryos [11]. The heart rate of 48 hpf zebrafish embryos were not significantly affected after exposure to 1 μM of Cd [23]. In addition, Johnson et al. [24] found that exposure to 0.828 μM Cu did not induce the developmental inhibition (death rate, hatching rate, body length, and heart rate) of zebrafish embryos, while ≥1.45 μM of Cu could induce a significantly higher heart rate for zebrafish embryos. However, the heart rate of zebrafish embryos exposed to 4.25 mM CIP for 4 h was significantly reduced due to the extremely high CIP concentration [10].

Similarly, no significant effects on the death rate, hatching rate, spontaneous movement, and heart rate of zebrafish embryos were observed in the binary mixtures of Cu + ENR, Cu + CIP, Cd + ENR, and Cd + CIP (Fig. 2). Therefore, both single pollutant and mixture exposures had insignificant developmental toxicity to zebrafish embryos, probably due to the low exposure concentrations in this study (≤0.6 μM). Willi et al. [25] also observed that exposure to 0.056 nM and 0.561 nM mixtures of androgens and progestins had no observed effects (spontaneous muscle contractions, heart rate, edema, and paralysis) on zebrafish embryos, but altered the transcriptional expressions of genes (cytP19b, sult2s3, and cytP2K22). Thus, it is possible that molecular responses may be triggered by the low concentrations of Cu, Cd, ENR, and CIP individually or in mixtures, despite the insignificant developmental toxicity to zebrafish embryos.

3.2. Oxidative stress, inflammation, and lipid depletion in zebrafish larvae

The in vivo fluorescence images of zebrafish larvae showed that the production of ROS was promoted when exposed to Cu, Cd, ENR, or CIP individually, compared with the control group (Fig. 3a), and the increase of ROS depends on the concentration of the chemicals (Figs. S3a and S3b). Previously, it was reported that the oxidative stress was induced when zebrafish embryos were exposed to 0.1 μM Cu or 0.08 μM Cd [26], and erythrocyanotes to 0.139 μM ENR or 0.151 μM CIP [27]. In the single exposure groups, Cd induced greater ROS production than Cu (Fig. 3d). Moreover, in general, exposure to metals resulted in higher ROS production than exposure to FQs (Fig. 3d). The increase in ROS production indicates that oxidative stress occurred in zebrafish larvae. Moreover, ROS can target DNA, lipids, and proteins, and consequently affect cell physiological pathways, signaling cascades, and membrane properties, which ultimately cause inflammation and cell death [28].

In the combined exposure groups, the addition of ENR or CIP decreased the ROS content in zebrafish larvae, compared with the larvae exposed to Cu or Cd individually (Fig. 3d). This antagonistic effect of ENR and CIP on the oxidative stress induced by Cu or Cd in zebrafish was intriguing and probably resulted from the complexation of Cu and Cd with ENR and CIP (Text S5) and subsequent masking to ROS generation.

Macrophages are an important part of the innate immune system, which are recruited to the inflammatory sites to participate in nonspecific (innate immunity) and specific defense (cellular immunity) [29,30]. Macrophages in zebrafish larvae were apparently elevated in all exposure groups compared with the control group (Fig. 3b and e). Moreover, the increase of macrophages in zebrafish larvae was more pronounced in the combined exposure groups than in the single exposure groups. The results suggest that exposure to environmental concentrations of Cu, Cd, ENR, and CIP individually can cause inflammation in zebrafish larvae, and the inflammation response was aggravated when exposed to the metal–antibiotic mixtures.

The fluorescence intensity of Nile red in zebrafish larvae decreased in all the exposure groups (Fig. 3c and f), indicating that exposure to single or mixed metals and FQs reduced lipid content (or increased the lipid metabolism) in zebrafish larvae. Moreover,
the decrease in lipid content in the combined exposure groups was greater than that in the single exposure groups. Similarly, previous studies also reported that exposure to Cd decreased the lipid content in crab, eel, and aquatic birds [31–33]. This may be due to the enhanced mitochondrial metabolism and the decreased synthesis of fatty acids [31].

3.3. Bioaccumulation of metals in zebrafish embryos

The reduced Cu and Cd in exposure solutions increased with increasing initial metal concentrations (Fig. 4a and b), correspondingly resulting in an increased amount of Cu and Cd in zebrafish larvae (Fig. 4e and f). Notably, in the combined exposure groups, the amounts of Cu and Cd both reduced in exposure solutions and accumulated in zebrafish larvae increased with increasing concentration of ENR or CIP (Fig. 4c, d, g, and h). Thus, the addition of ENR or CIP promoted the uptake of metals by zebrafish embryos. This might be due to the uptake of metal–FQs complexes in addition to uptake of uncomplexed metals and/or a change in uptake and elimination kinetics [34]. Indeed, the complexation of Cu and FQs occurred in the present study (Text S5, Fig. S1, and Fig. S2). Similarly, it was reported that the presence of ENR increased the absorption of Cd by Eisenia fetida due to the complexation between them [34]. Chlortetracycline facilitated the intracellular uptake of Cu and Cd by increasing the membrane permeability of Synecochlorella. Moreover, the number of reduced metals in the exposure solution was much more than the metals in zebrafish larvae, suggesting that most of the metals (73–95%) may be accumulated in the embryo’s chorion. Wang et al. [36] also found that more than 95% of Cu was accumulated in the chorion rather than in the embryo due to its strong adsorption to metals.

3.4. Metabolite responses to the exposure of metals or/and FQs

In the metabolomics analysis, a total of 10644 peaks were detected in all the exposure groups, and 4587 were selected as DEMs. Volcano plots showed that the numbers of DEMs in the combined exposure groups (Fig. 5 f-i) were higher than those in the
single exposure groups (Fig. 5a–d and j). Thus, exposure to the mixture of metals and FQs could more strongly disturb intracellular metabolites in zebrafish larvae than their single exposure. The stronger interference in the combined exposure groups was further confirmed by OPLS-DA (Fig. 5e), which showed a farther distance between the binary exposure groups and the control group than those between the single exposure groups and the control group. This could be explained by the increased bioaccumulation of Cu and Cd in zebrafish larvae in the binary exposure groups (Fig. 4g and h).

Among 4587 DEMs, only 385 DEMs were qualitatively identified. The identified DEMs mainly include organic acids and their derivatives (29.6–66.0%), lipids and lipid-like molecules (18.1–42.0%), organoheterocyclic compounds (6.3–13.0%), and nucleosides, nucleotides and analogs (4.0–6.6%) (Fig. S4). Among them, organic acids and derivatives and lipids and lipid-like molecules were the most detected in all exposure groups. Furthermore,
the majority of the organic acids and derivatives were up-regulated (Fig. S5), whereas most of the lipids and lipid-like molecules were down-regulated in various exposure groups (Fig. S6).

### 3.4.1. Organic acids and derivatives

Most amino acids, as the majority of organic acids and derivatives, were upregulated in all the exposure groups (Fig. S5). Moreover, the upregulation of amino acids in the mixture exposure groups was stronger than in the single exposure groups. Thereinto, glycine, glutamic acid, and serine are utilized to generate glutathione (GSH), which has antioxidant activity and detoxification effects, and plays an important role in the immune system [37,38]. The up-regulation of these metabolites (Fig. 6a) implies the active responses of zebrafish larvae to external stimuli [11,39]. Moreover, the previous study has reported that metal ions are highly chelated with some amino acid (i.e., threonine, isoleucine, and serine) through strong covalent bonds [40], thus controlling the accumulation and release of metal ions in organisms. For example, when *Synechocystis* sp. exposed to Cu, the sequestration proteins bound to the extracellular Cu, and thus inhibited the excessive Cu uptake [35]. Cd stress induced the production of metallothionein protein by *Eisenia fetida* effectively, detoxifying Cd through binding it in the tissue [34].

Lactate has been used as a marker of inflammation [41]. Glutamine plays an important role in reducing inflammation by providing fuel for T-cell [42]. Moreover, glycine also has anti-inflammatory and immunomodulatory effects via suppressing the activation of transcription factors, the formation of free radicals,
and the production of inflammatory cytokines [43]. Therefore, the up-regulation of lactate, glutamine, and glycine (Fig. 6a) indicates the occurrence of inflammation in zebrafish larvae. In addition, the ratio of kynurenine to tryptophan (Kyn/Trp) has been widely used as a biomarker indicating cytokines (interferon-gamma) mediated inflammatory activity [44]. Previous studies have reported the increase of Kyn/Trp ratio was positively correlated with inflammation and inflammation-related diseases [45,46]. The Kyn/Trp in all exposure groups also significantly increased (Fig. 6c), indicating the occurrence of inflammation and immune activation [47].

### 3.4.2. Lipids and lipid-like molecules

Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and oleic acid were significantly down-regulated, and the degree of down-regulation in the combined exposure groups was greater than those in the single exposure groups (Fig. 6b). As EPA, DHA, DPA, and oleic acid can be used to produce specialized pro-resolving lipid mediators (SPM) [48], their down-regulation could decrease SPM production. Because SPM plays a role in hindering the production of pro-inflammatory cytokines and prevents chronicity of inflammation and/or autoimmunity [49], the lower SPM production may facilitate inflammation. Moreover, recent evidence indicates that insufficient production of SPM can trigger chronic inflammation, which is associated with several chronic diseases, such as chronic obstructive pulmonary disease, type-2 diabetes, and rheumatoid arthritis [49–51]. In addition, EPA and DHA, mainly found in fatty fishes, have health benefits such as anti-arrhythmic, hypolipidemic, and anti-thrombotic effects [52,53]. Thus, our results suggest that exposure to metals or/and FQs may reduce the nutritional value of freshwater fish.

Lysophospholipids (LysoPLs), including lysophosphatidylcholine (LysoPC) and lysophosphatidyl ethanolamine (LysoPE), were down-regulated in all the exposure groups (Fig. 6d). LysoPC had anti-inflammatory effects under various pathological conditions [54], and the decrease of LysoPC was associated with many chronic inflammation-related diseases such as rheumatoid arthritis [55], type-2 diabetes [56], and liver cirrhosis [57]. Furthermore, the decrease in LysoPC levels is considered a potential biomarker of cancer [58]. On the other hand, LysoPE can stimulate chemotactic migration and cellular invasion in cancer by activating the mitogen-activated protein kinase (MAPK) signaling [59,60]. Therefore, the down-regulation of LysoPC and LysoPE may induce inflammation and even increase the risk of cancer.

### 3.5. Protein responses to the exposure of metals or/and FQs

A total of 4404 proteins were qualitatively detected by the proteomics analysis, and 1035 DEPs were identified in all the exposure groups. The number of DEPs induced by Cd (464) was higher than that by Cu (169). The number of DEPs induced by ENR (448) was higher than that by CIP (103) (Fig. 7a–d). The results are in agreement with their ROS production in zebrafish larvae (Fig. 3d and e). Exposure to Cd or ENR induced more DEPs than other single and combined exposure groups (Fig. 7a–d). Additionally, when comparing the protein responses of the combined exposure groups with their corresponding single exposure groups, no consistent patterns could be found, which may be due to complex up- and down-regulation of the myriad of proteins in response to pollutant exposure as a function of exposure duration and physiological conditions (such as growth stage) [61]. This ambiguity on the relationship of exposure and protein patterns points to a challenge in proteomics, which should be further investigated.

#### 3.5.1. GO functional classification of DEPs

According to the GO functional classification (Fig. S7 and Fig. 7e and f), many proteins related to response to stimulus and immune system process were dysregulated significantly in all the exposure groups. This corresponds to the occurrence of inflammation and possible tumorigenesis in zebrafish larvae, which was also corroborated by the metabolomics analysis (Fig. 6). Moreover, corresponding to the increase of ROS content (Fig. 3a and d), the typical antioxidant enzymes, such as superoxide dismutase (sod1), glutathione S-transferase (gstp1), and peroxiredoxin (prdx1 and prdx6), were up-regulated in most of the exposure groups (Fig. 7g), indicating the active response of zebrafish larvae to oxidative stress [22].

Additionally, the majority of the typical immune proteins, such as lysozyme (lyz), tumor necrosis factor receptor-associated protein (trap1), two subtypes of heat shock protein 90 (hsp90), hsp90a.1 and hsp90ab1, and migration inhibitory factor (mif) were up-regulated in all the exposure groups (Fig. 7h). Among them, lyz,
produced by leukocytes, is an essential part of innate immunity in fish [62]. Trap1 could promote inflammation by regulating the activation of proinflammatory ligands [63]. Hsp90 is vital to maintain cellular homeostasis [64], and the overexpression of hsp90 leads to enhanced proliferation, migration, invasion, and tube cell-dependent tumor angiogenesis in vitro and in vivo [65]. The protein of macrophage mif could induce the inflammatory reaction by inhibiting the migration of macrophages, sustaining macrophage viability, and stimulating the expression of the proinflammatory cytokines [66,67]. Moreover, mif potentially promotes tumorigenesis by inhibiting the classic tumor suppressor gene p53 [68]. Therefore, the up-regulation of the typical immune proteins further confirms the occurrence of inflammation and may also suggest a potential risk of tumorigenesis.

3.5.2. KEGG pathway analysis

KEGG pathway enrichment analysis showed that many pathways related to tumor and immunity were significantly altered (Fig. 8a). Among the enriched pathways related to immunity, three chronic disease pathways (inflammatory bowel disease, systemic lupus erythematosus, and rheumatoid arthritis) were associated with chronic inflammation, suggesting the occurrence of inflammation. Among the enriched pathways related to the tumor, the PI3K-Akt signaling pathway is vital to the growth and survival of tumor cells [69]. Focal adhesion, associated with the development of malignancy perturbations, plays an important role in the proliferation, survival, and migration of cells [70]. Extracellular matrix (ECM)-receptor interaction is critical to the formation of breast tumor, pancreatic tumor, neural tumor, and liver tumor [71–74] by regulating cellular survival, proliferation, differentiation, shape, polarity, and motility [75]. These three pathways were significantly dysregulated in most exposure groups except for CIP. Taken together, the results suggest that exposure to FQs or metals caused significant changes in the pathways related to immunity and even might increase the potential risk of tumorigenesis in zebrafish larvae.

A total of 55 DEPs were involved in the pathways related to the tumor, and they can be classified into 3 major groups by the protein-protein interaction (PPI) network analysis (Fig. 8b). Group 1 composed 14–3–3 proteins (encoded by ywha genes) and mapk. The 14–3–3 proteins are regulators of intracellular signaling pathways targeting oncogenic proteins [76], and mapk is a complex interconnected signaling cascade with frequent involvement in the initiation and progression of the tumor [77]. Moreover, the 14–3–3 proteins can activate mapk [76]. Group 2 consisted of collagens (encoded by col-genes), fibronectin (encoded by fn1a-genes), and laminin (encoded by lama5a-genes), and they are the major components of the ECM [78]. Fibronectin and laminin provide the crucial connections for the interaction of other ECM components (collagens) and cells, which may mediate tumorigenesis [75]. The major components of Group 3 were glutathione S transferase (encoded by gst-genes) and mapk. High levels of glutathione S transferase are closely related to oxidative stress induced by ROS [22] and can promote cancer cell survival [79]. Moreover, ROS could activate the downstream target mapk proteins [80].

Notably, PPI network analysis also revealed that Group 1 was closely related to Group 2 and Group 3, but no relation was observed between Group 2 and Group 3. The 14–3–3 proteins in...
Group 1 could participate in the adhesion of cells and ECM (fibronectin and laminin in Group 2), which were the key proteins linking Group 1 with Group 2 (Fig. S8). The adhesion of tumor cells may contribute to tumorigenesis [81]. Group 3 was related to Group 1 through the interactions between different mapk proteins involved in these two groups. The mapk pathway is activated through the cascade of different mapk proteins [77] in the order of map2k2a in Group 1 and mapk8 and mapk10 in Group 3 (Fig. S8), resulting in the close relationship between these two groups.

In addition to the DEPs involved in the pathways related to the tumor, some ribosomal L (rpl), keratin (krt), and fatty acid-binding protein (fabbp) also play important roles in tumorigenesis [82–84]. For example, the tumor suppressors, the proteins encoded by rpl36, rpl36a, rpl23, and rpl23a [84], were down-regulated in the majority of the exposure groups (Fig. 8c). This could enhance the tumor susceptibility [85]. Krt4 and krt5 are key genes that regulate lipoma and glioma, respectively [82,83], and the proteins encoded by these genes were significantly upregulated by the exposure to ENR and the Cd + ENR mixture, which may lead to the formation of lipoma and glioma (Fig. 8c). Fabp2, fabp3, fabp7a, and fabp10a are vital to the development of hepatocellular carcinoma [86], and the up-regulation of the proteins encoded by these genes implies the increased likelihood of hepatic tumorigenesis when exposed to environmental concentrations of metals and antibiotics (Fig. 8c).

4. Conclusion

This study investigated the developmental toxicity of FQs (ENR and CIP) and heavy metals (Cu and Cd), individually and in combination, toward zebrafish embryos. The molecular responses of zebrafish larvae to these pollutants were explored by the metabolomics and proteomics analysis. Exposure to single and binary mixture of FQs and metals did not cause any developmental toxicity to zebrafish embryos, but induced oxidative stress, inflammation, and lipid depletion. Moreover, the inflammation and lipid depletion were more severe in the mixture exposure groups than in the single exposure groups, probably due to the increased metal bioaccumulation in the presence of ENR or CIP. We observed inflammation and potential biomarkers of tumorigenesis in zebrafish larvae by using metabolomics and proteomics analysis, when the zebrafish embryos were exposed to a no-observed-effect concentration of FQs and metals. The findings highlight the potential toxicological risks of pollutants at environmentally relevant trace levels, which may be overlooked in the acute toxicity tests. Moreover, FQs and heavy metals are ubiquitous in natural waters, and thereby their potential ecological and human health risks should not be ignored. This study was limited to the early development stage of zebrafish (≤96 hpf). In a natural aquatic ecosystem, chronic exposure at low pollutant concentrations might further contribute to the complexity and uncertainty in the toxicity of pollutant mixtures. Therefore, future studies examining the long-term toxicological effects of environmental concentrations of pollutants are encouraged.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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