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Individual and joint toxic effects of cadmium sulfate and α-naphthoflavone on the development of zebrafish embryo*

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Abstract: This paper aims to evaluate the individual and joint toxicities of cadmium sulfate ($CdSO_4$) and α-naphthoflavone (ANF) in zebrafish embryos. As a result, $CdSO_4$ caused both lethal and sub-lethal effects, such as 24 h post-fertilization (hpf) death and 72 hpf delayed hatching. However, ANF only caused sub-lethal effects, including 48 hpf cardiac edema and 72 hpf delayed hatching. Taking 24 hpf death and 48 hpf cardiac edema as endpoints, the toxicities of $CdSO_4$ and ANF were significantly enhanced by each other. Consistently, both $CdSO_4$ and ANF caused significant oxidative stress, including decreases in the reduced glutathione (GSH) level, inhibition of superoxide dismutase (SOD) activity, as well as increases in malondialdehyde (MDA) content in zebrafish embryos, but these mixtures produced much more significant alterations on the biomarkers. Co-treatment of $CdSO_4$ and ANF significantly down-regulated the mRNA level of multidrug resistance-associated protein (*mrp*) 1 and cytochrome P450 (*cyp*) *1a*, which constituted the protective mechanisms for zebrafish embryos to chemical toxins. In conclusion, co-treatment of $CdSO_4$ and ANF exhibited a much more severe damage in zebrafish embryos than individual treatment. Meanwhile, production of oxidative stress and altered expression of *mrp1* and *cyp1a* could be important components of such joint toxicity.

Key words: Joint toxicity, Cadmium sulfate, α-Naphthoflavone, Zebrafish embryo, Oxidative stress
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1 Introduction

Aquatic organisms are usually faced with various types of chemical toxicants in the water, and thus have to suffer from the unpredictable interactions of these mixtures (Duan *et al.*, 2008; Maria and Bebianno, 2011). Unfortunately, although there have been numerous investigations on the toxicity of a specific compound (Shi *et al.*, 2008; Osterauer *et al.*, 2011), information regarding the joint effects of pollutants remains insufficient.

Cadmium is one of the most important heavy metal toxicants and its environmental concentration is increasing due to its extensive utilization in modern industries. Cadmium is reported to cause many deleterious effects including impaired neurogenesis, eye defects, and hatching failure in aquatic organisms (Chow *et al.*, 2008; Matović *et al.*, 2011). Various experiments have been done on zebrafish, *Sparus aurata*, and gilthead sea bream larvae, and the results revealed that the toxicity of cadmium was caused by the inhibition of cytochrome P450 (CYP) 1A expression as well as the production of oxidative stress (Sassi *et al.*, 2013; Souid *et al.*, 2013; Wang and Gallagher, 2013).

Polycyclic aromatic hydrocarbon (PAH), on the

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other hand, consists of a large number of compounds with different structures, and is increasingly released by oil leakages, vehicle emissions, and agriculture/industry activities. In aquatic systems, PAHs like benzo[α]pyrene and retene pose significant threats, such as pericardial edema, craniofacial malformations, and altered development of visual systems in fishes (Fleming and di Giulio, 2011; Hawliczek *et al.*, 2012; Huang *et al.*, 2014). And such threats are reportedly related to the metabolism by CYP1A as well as enhanced levels of oxidative stress (Timme-Laragy *et al.*, 2007).

Adenosine triphosphate-binding cassette (ABC) transporters, such as multidrug resistance-associated protein (Mrp) 1–5 genes, have also been considered to be involved in the detoxification of cadmium and PAH. High expression and function of these transporters have been found in fishes, such as zebrafish (Long *et al.*, 2011a; 2011b) and rainbow trout (Kennedy *et al.*, 2014). Their gene expression could be induced by various xenobiotics like cadmium and PAH, pumping the parent compound and their metabolites out of the organisms in an energy-dependent process (Long *et al.*, 2011c; Costa *et al.*, 2012; Navarro *et al.*, 2012). Thus, ABC transporters are usually considered to be the major biological defense mechanism for the protection of organisms against these environmental chemicals.

Due to their wide applications, cadmium and PAH usually coexist in the environment (Zhang *et al.*, 2004; Keenan *et al.*, 2010), and interactions of these chemicals in fishes might happen due to the similar induction of oxidative stress and the involvement of CYP1A and ABC transporters. However, such interactions have never been studied in previous experiments. In this paper, zebrafish embryos, which represented an attractive model for studying the toxic mechanisms of environmental chemicals (Berry *et al.*, 2007; Weil *et al.*, 2009), were used to examine the individual and joint effects of cadmium sulfate ($CdSO_4$) and a model PAH, α-naphthoflavone (ANF) (Timme-Laragy *et al.*, 2007). To assess the possible role of oxidative stress, ABC transporters and CYP1A in the joint toxicity, the alteration of reduced glutathione (GSH) level, superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, and *mrp1* and *cyp1a* expression caused by $CdSO_4$, ANF, and $CdSO_4$-ANF mixtures were respectively evaluated.

2 Materials and methods

2.1 Reagents

Both $CdSO_4$ and ANF were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and their purity was above 99%. Kits for GSH level, SOD activity, and MDA content were obtained from Beyotime Institute of Biotechnology (Nantong, China). The other reagents were of reagent grade and purchased from local suppliers.

2.2 Zebrafish

Adult wide-type zebrafish were purchased from a local aquarium (Suzhou, China) and maintained as described before (Berry *et al.*, 2007). Fish eggs were collected and washed three times with Holtfreter's buffer (3.5 g/L NaCl, 0.05 g/L KCl, 0.1 g/L $CaCL_2$, and 0.025 g/L $NaHCO_3$; pH 7.5). Using an Axio Observer A1 microscope (Carl Zeiss, Inc., Oberkochen, Germany), the status of the collected eggs was visually determined, and any dead eggs were discarded. At 4 h post-fertilization (hpf), the good-quality eggs were collected and placed in a 24-well plate such that each well contained 10 embryos, and were used for developmental toxicity assays (Tilton and Tanguay, 2008).

2.3 Developmental toxicity test

$CdSO_4$ was dissolved in Holtfreter's buffer directly before use. ANF was dissolved in acetone first and then diluted in Holtfreter's buffer. In each treatment, the concentration of acetone was no more than 0.1%, which caused no significant alteration in the development of embryos.

At 4 hpf, eggs in 24-well plates were washed with Holtfreter's buffer and exposed to 2 ml Holtfreter's buffer containing the vehicle, $CdSO_4$, ANF, or $CdSO_4$-ANF mixtures. Each group contained at least six replicates, and eggs treated by 0.1% acetone acted as vehicle controls. Moreover, transparent plastic film was used to cover the wells. Half of the exposure solutions of each well were removed and then were replaced daily with fresh exposure solutions. Meanwhile, the dead animals were removed. At 24, 48, and 72 hpf, the developmental status of the zebrafish embryos treated by vehicle control (Figs. 1a and 1c) and chemicals (Figs. 1b and 1d) was observed with the microscope and documented using a A2000IS

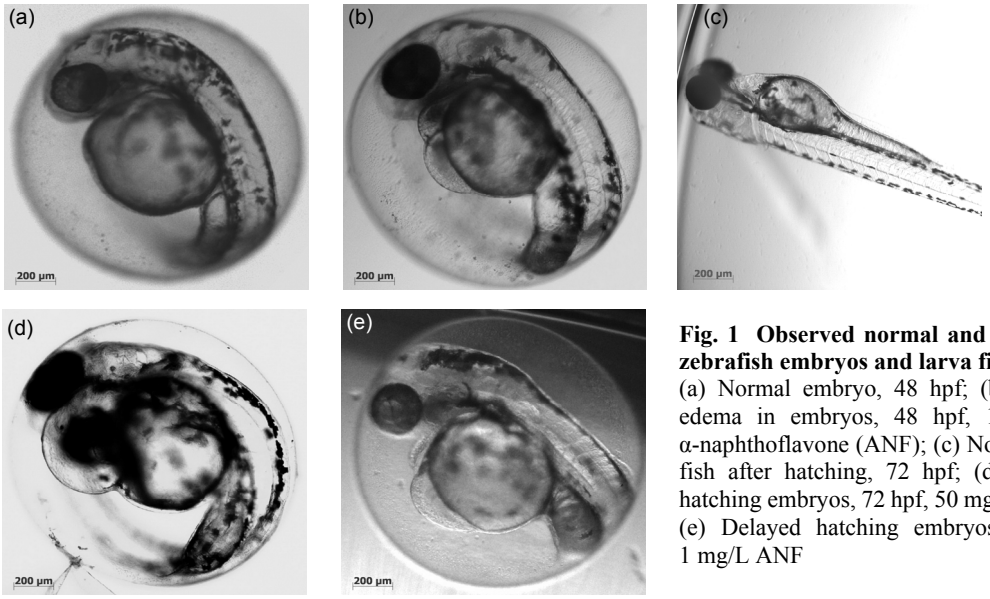

Fig. 1 Observed normal and abnormal zebrafish embryos and larva fish
(a) Normal embryo, 48 hpf; (b) Cardiac edema in embryos, 48 hpf, 1.50 mg/L α-naphthoflavone (ANF); (c) Normal larva fish after hatching, 72 hpf; (d) Delayed hatching embryos, 72 hpf, 50 mg/L $CdSO_4$; (e) Delayed hatching embryos, 72 hpf, 1 mg/L ANF

camera (Conan Co., Ltd., Beijing, China). The selected endpoints included 24 hpf death, 48 hpf cardiac edema (Fig. 1b), and 72 hpf delayed hatching (Figs. 1d and 1e).

To more specifically identify the effect of ANF on the toxicity of $CdSO_4$, we also conducted an experiment in which embryos were first treated by 75 mg/L $CdSO_4$ alone, and then 12 h later, the embryos were washed with Holtfreter's buffer and exposed to the mixtures of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF. Mortalities of zebrafish embryos after 20 h treatment (16–36 hpf) were subsequently recorded and compared with groups treated only by $CdSO_4$ (4–36 hpf) or the simultaneous treatment of $CdSO_4$ and ANF (4–24 hpf).

2.4 GSH, MDA, and SOD detection

At 24, 36, and 48 hpf, three sets of 60 embryos in both the vehicle control and chemical treatment groups were washed with Holtfreter's buffer and collected into 1.5 ml centrifuge tubes. Each tube was filled with 300 μl phosphate buffer solution (PBS; pH 7.4) and immersed in liquid nitrogen for 20 s. After that, the embryos in PBS were mechanically homogenized (Wiegand *et al.*, 1999). The supernatant was collected after the centrifugation of the embryo homogenate (10000×*g*, 4 °C, 10 min) for a biomarkers assay.

GSH level, SOD activity, and MDA content were detected using commercially available kits. The protein content of supernatants was detected using the bicinchoninic acid method (Walker, 1994). The GSH level was determined via the formation of 5-thio-2-nitrobenzoic acid (412 nm) (Hao *et al.*, 2013). SOD activity was detected by the nitroblue tetrazolium/riboflavin photometric quantitative methods (420 nm) (Janknegt *et al.*, 2007). The content of MDA was detected using the thiobarbituric acid assay (535 nm) (Dong *et al.*, 2013). All the biomarker detections were conducted with the Synergy HT multi-mode microplate reader (Bio-Tek Instruments, Inc., Vermont, USA). After this, all the biomarkers were normalized to the total protein content and expressed as a percentage of the groups treated by the vehicle.

2.5 Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of *cyp1a* and *mrp1*

At 24 and 48 hpf, zebrafish embryos treated by the vehicle, 75 mg/L $CdSO_4$, 1.5 mg/L ANF, or the mixtures of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF were collected. The living ones were used to extract total RNA with a commercial kit (Axygen Scientific, Inc., USA). The RNA quality was checked by 260/280 nm absorption using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). First-strand cDNA was prepared as previously described by Nakashima *et al.* (2012). After this, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), *mrp1*, and *cyp1a* were amplified from the first-strand cDNA using the PCR. Among these, *gapdh* was set as the house keeping gene. Gene primer sequences of *gapdh*, *mrp1*,

and *cyp1a* (Table 1) were designed using Primer Premier 5.0 software (Premier, Inc., Canada).

2.6 Statistical analysis

All of the experimental data were expressed as mean±standard deviation (SD) of three sets of independent experiments, which were subsequently analyzed using a Statistical Package for Social Sciences (SPSS) v.15.0 (Chicago, USA). One-way analysis of variance (ANOVA) for multiple groups and Tukey's HSD tests for two different treatments were performed to compare the data, respectively. In all cases, data differences were statistically significant when $P<0.05$.

3 Results

3.1 Individual toxicities of $CdSO_4$ and ANF

As shown in Figs. 1 and 2, zebrafish embryos treated by the vehicle control (0.1% acetone) exhibited no significant alteration. $CdSO_4$ revealed both lethal and differential sub-lethal effects, like 24 hpf death and 72 hpf delayed hatching (Fig. 1d). However, only sub-lethal effects, such as 48 hpf cardiac edema (Fig. 1b) and 72 hpf delayed hatching (Fig. 1e) were observed for ANF. Furthermore, concentration-dependent toxic effects of both toxic compounds were obtained and are shown in Fig. 2. For example, the mortality of the embryos at 24 hpf was (10.00±4.61)% after a treatment of 100 mg/L $CdSO_4$, while, all embryos were dead at 24 hpf when exposed to 300 mg/L $CdSO_4$. The treatment of 20 mg/L $CdSO_4$ caused a delayed hatching rate of (17.50±5.00)% at 72 hpf, while the delayed hatching rate increased to 100% when the embryos were treated by 75 mg/L $CdSO_4$. For ANF, a similar trend was also found. For example, exposure to 1 mg/L ANF resulted in a cardiac edema rate of (26.25±10.15)% at 48 hpf, while nearly all embryos had cardiac edema at 48 hpf after the treatment of 3 mg/L ANF.

3.2 Joint toxicity of $CdSO_4$ and ANF

Since 24 hpf death only occurred in embryos treated by $CdSO_4$ and 48 hpf cardiac edema in embryos was only observed after the treatment of ANF, they were selected as endpoints for the investigation of the joint toxicity of $CdSO_4$ and ANF. As a result, toxicities of $CdSO_4$ and ANF could be significantly enhanced by each other (Fig. 3). For example, the treatment of 75 mg/L $CdSO_4$ or 1.5 mg/L ANF alone did not cause any death of zebrafish embryos, but co-treatment of 1.5 mg/L ANF and 75 mg/L $CdSO_4$ resulted in a 24 hpf mortality of (31.11±6.12)%. Furthermore, all embryos were dead at 24 hpf after exposure to the co-treatment of 1.5 mg/L ANF and 150 mg/L $CdSO_4$, while they were all alive when treated by $CdSO_4$ or ANF alone ($P>0.05$). Similarly, the treatments of 1.5 mg/L ANF and 75 mg/L $CdSO_4$ resulted in a 48 hpf cardiac edema rate of (58.33±12.93)% and 0, respectively, but the cardiac edema rate was (85.00±4.25)% when embryos were treated by a combination of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF ($P<0.001$).

3.3 Altered oxidative stress state of zebrafish embryos by the individual treatments of $CdSO_4$ and ANF

Generally, $CdSO_4$ and ANF caused a concentration- and time-dependent alteration of the GSH level, SOD activity, and MDA content (Fig. 4). For example, SOD activity at 24 hpf was unaffected by 100 mg/L $CdSO_4$, but it was reduced by 9% when treated by 200 mg/L $CdSO_4$. Meanwhile, the reduction of SOD activity at 48 hpf was about 25% when embryos were treated by 200 mg/L $CdSO_4$ (Figs. 4a and 4b). Besides, the effects of ANF on GSH level, SOD activity, and MDA content of embryos at 48 hpf were not

Table 1 Specific primer sequences used in this experiment

Gene	Primer sequence (5'→3')	Product size (bp)	Reference
gapdh	Sense: GCAACACAGAAGACCGTTGA	440	NM_001115114.1
	Anti-sense: GCCATCAGGTCACATACACG		
mrp1	Sense: TTGGATGGAGCTGGGTTTCC	437	XM_001341859.4
	Anti-sense: CTGAACTGCCACCTCGCTTA		
cyp1a	Sense: TGATGGAAAGAGTCTGGCGT	457	NM_131879.1
	Anti-sense: CTCCATCACCAGCCTCTTCA		

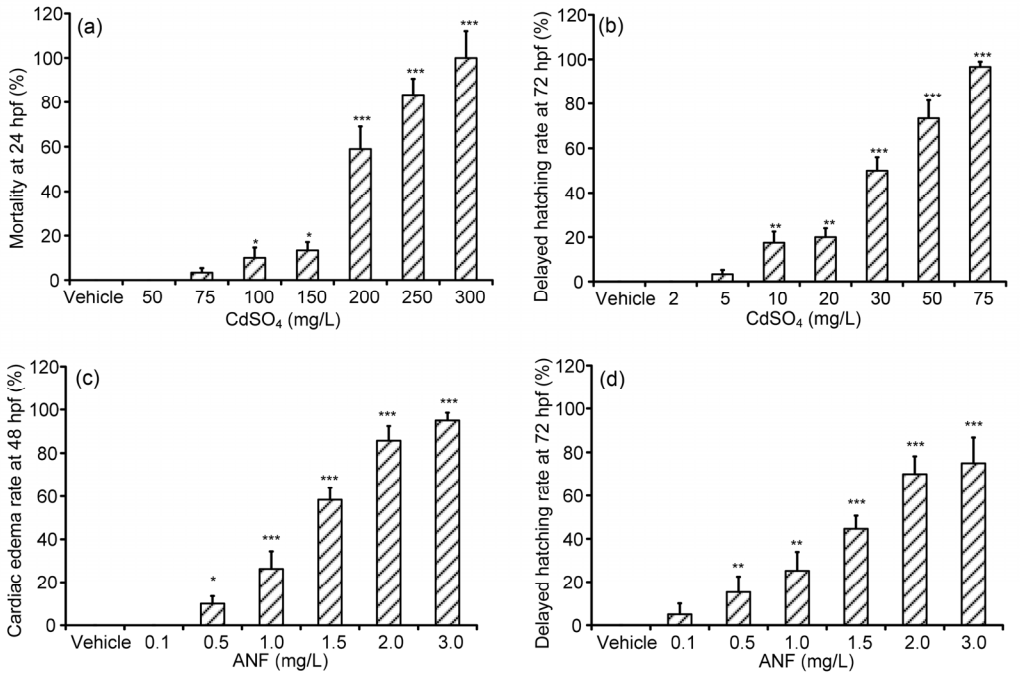

Fig. 2 Concentration-response relations for cadmium sulfate ($CdSO_4$) and α-naphthoflavone (ANF) alone
(a) Mortality at 24 hpf, $CdSO_4$; (b) Delayed hatching rate at 72 hpf, $CdSO_4$; (c) Cardiac edema rate at 48 hpf, ANF; (d) Delayed hatching rate at 72 hpf, ANF. Cardiac edema rate is the percentage of embryos having cardiac edema; delayed hatching rate is the percentage of embryos which exhibited delayed hatching. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the vehicle control. Values are expressed as mean±SD of three sets of independent experiments

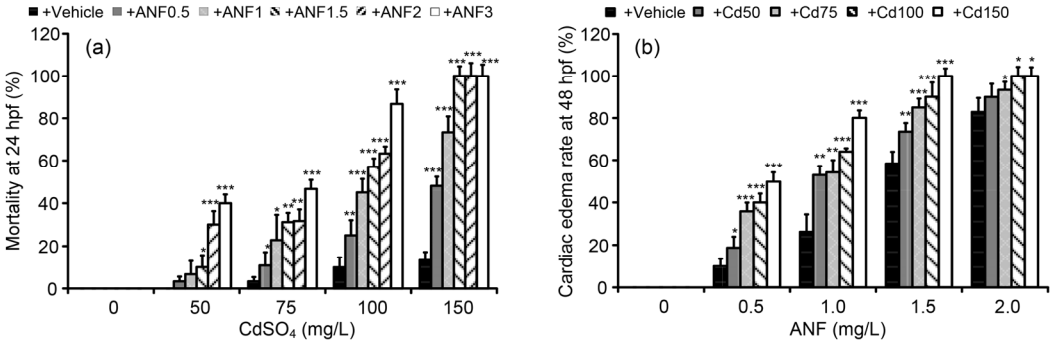

Fig. 3 Concentration-response relations for the combination of cadmium sulfate ($CdSO_4$) and α-naphthoflavone (ANF)
(a) Mortality at 24 hpf; (b) Cardiac edema rate at 48 hpf. Cardiac edema rate is the percentage of embryos having cardiac edema. ANF0.5, ANF1, ANF1.5, ANF2, and ANF3: 0.5, 1.0, 1.5, 2.0, and 3.0 mg/L ANF; Cd50, Cd75, Cd100, and Cd150: 50, 75, 100, and 150 mg/L $CdSO_4$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the groups treated by $CdSO_4$ (a) or ANF (b) only. Values are expressed as mean±SD of three sets of independent experiments

significant unless a concentration of 2 mg/L was used (Figs. 4c and 4d).

3.4 Altered oxidative stress state of zebrafish embryos by the co-treatment of $CdSO_4$ and ANF

Consistent with the joint toxicity of $CdSO_4$ and ANF, the mixtures produced great oxidative stress, including decreases in the GSH level, inhibition of SOD activity, and increase in MDA content, whereas no significant effects on the biomarkers were induced by the individuals at the corresponding doses (Fig. 5). For example, the SOD activity in embryos at 48 hpf was nearly completely inhibited by the co-treatment of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF, but it was unaffected by the individual treatment of either compound ($P>0.05$). Besides, the alteration in this biomarker was more pronounced with an increasing exposure period.

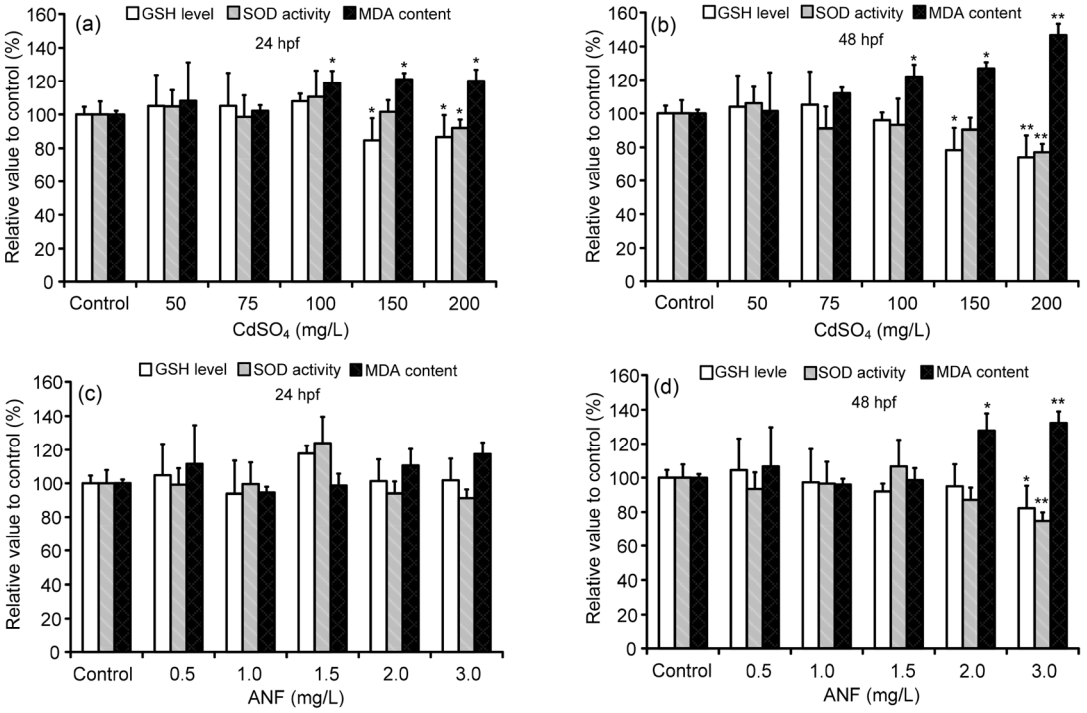

Fig. 4 Changes of oxidative stress state in zebrafish embryos at 24 hpf (a, c) and 48 hpf (b, d) upon exposure to cadmium sulfate ($CdSO_4$) (a, b) and α-naphthoflavone (ANF) (c, d)

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the vehicle control. Values are expressed as mean±SD of three sets of independent experiments

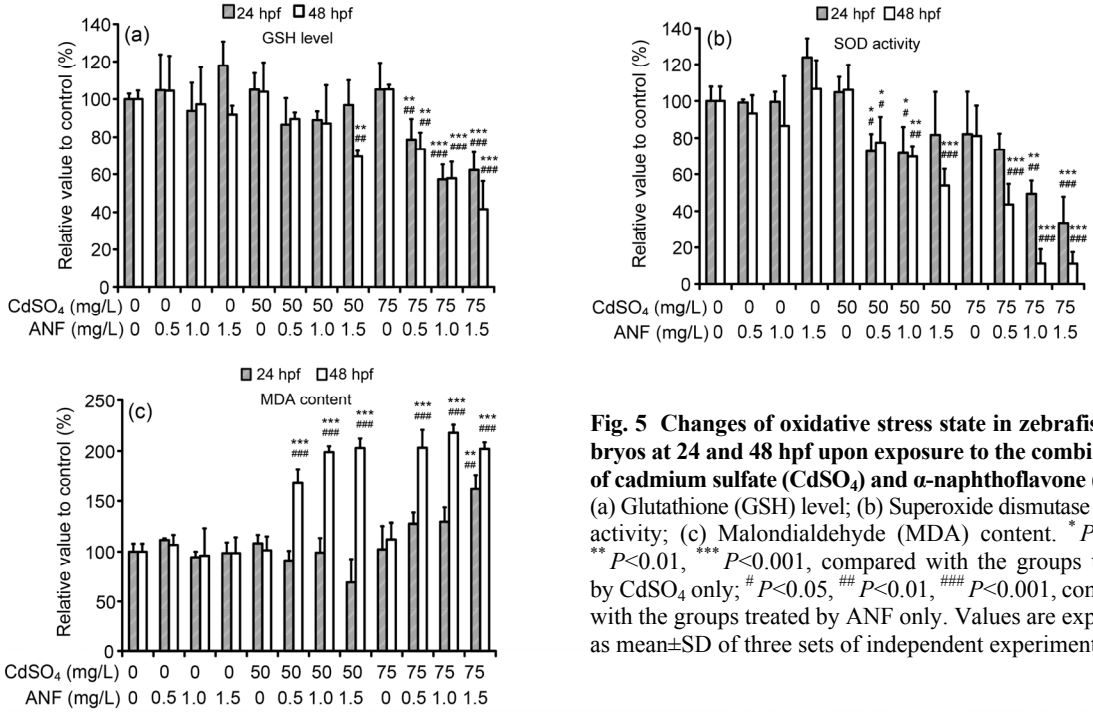

Fig. 5 Changes of oxidative stress state in zebrafish embryos at 24 and 48 hpf upon exposure to the combination of cadmium sulfate ($CdSO_4$) and α-naphthoflavone (ANF)

(a) Glutathione (GSH) level; (b) Superoxide dismutase (SOD) activity; (c) Malondialdehyde (MDA) content. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the groups treated by $CdSO_4$ only; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the groups treated by ANF only. Values are expressed as mean±SD of three sets of independent experiments

3.5 Toxicity of $CdSO_4$-ANF mixtures after a 12-h pre-treatment of $CdSO_4$

To more specially illustrate the mechanism of the interactions between $CdSO_4$ and ANF, we added 1.5 mg/L ANF 12 h after the pre-treatment of 75 mg/L $CdSO_4$ alone. As a result, such a treatment caused a much lower mortality and slighter alteration of an oxidative stress state than the simultaneous addition of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF (Table 2). For example, after 12 h pre-treatment of $CdSO_4$, the mixtures of $CdSO_4$ and ANF caused a reduction of SOD activity by 22%, while the simultaneous addition of $CdSO_4$ and ANF decreased the activity of SOD by about 67% ($P<0.05$). The addition of 1.5 mg/L ANF only slightly enhanced the toxicity compared with the group treated by 75 mg/L $CdSO_4$ alone. For instance, the 36 hpf mortality of embryos after the co-treatment of $CdSO_4$ and ANF was (15.23±2.04)%, which was only slightly higher than that of embryos treated by $CdSO_4$ alone ((3.96±4.19)%).

3.6 Alteration of *mrp1* and *cyp1a* gene expression

After the treatments of $CdSO_4$, ANF, and $CdSO_4$-ANF mixtures, mRNA expression levels of *mrp1* and *cyp1a* in zebrafish embryos at 24 and 48 hpf were respectively detected. And the results indicated that the individual treatments of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF both significantly induced the gene expression of *mrp1*, but the co-treatment of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF caused an obvious reduction of *mrp1* gene expression as compared with the vehicle controls. While for *cyp1a*, its gene expression was enhanced by ANF but inhibited by $CdSO_4$, and the co-treatment of $CdSO_4$ and ANF reduced the gene expression of *cyp1a* to near the level of the vehicle control. For both *mrp1* and *cyp1a*, the alteration of mRNA levels by chemicals seemed to be enhanced with incubation time.

4 Discussion

Aquatic organisms that inhabit environments are often contaminated with high levels of heavy metals and PAHs together (Terry and Stone, 2002; Udomchoke *et al.*, 2010). However, studies on the joint effects of these pollutants are still rare. In this respect, $CdSO_4$ and ANF were selected as the representatives of metals and PAH, and their joint toxicity and corresponding mechanisms were subsequently studied.

Both $CdSO_4$ and ANF exhibited significant developmental toxicity in zebrafish embryos (Figs. 1 and 2). Meanwhile, the toxicities of $CdSO_4$ and ANF could be significantly enhanced by each other as observed based on 24 hpf death and 48 hpf cardiac edema (Fig. 3). In the previous reports, glutathione *S*-transferase conjugation of GSH was considered to be important in the detoxification of cadmium in zebrafish embryos (Notch *et al.*, 2011), while the treatment of ANF has been reported to significantly inhibit the activity of glutathione *S*-transferase and the subsequent antioxidant response in zebrafish embryos (Gauthier *et al.*, 2014), indicating an interactive role of ANF with $CdSO_4$. The modulation of glutathione *S*-transferase by cadmium (Matović *et al.*, 2011; Yang *et al.*, 2012) might also result in the enhancement of ANF toxicity, which was related to the production of oxidative stress (Fleming and di Giulio, 2011). In this paper, both $CdSO_4$ and ANF caused decreases in the GSH level, inhibition of SOD activity, and increases in MDA content in zebrafish embryos (Fig. 4), but the mixtures produced a much greater oxidative stress (Fig. 5). Thus, oxidative stress should be considered important in the interaction of $CdSO_4$ and ANF.

Interestingly, the same concentration of $CdSO_4$-ANF mixtures produced a reduced toxicity when the embryos were pretreated by $CdSO_4$ for 12 h (Table 2), which should be due to the induction of the detoxification system by the pretreatment of $CdSO_4$ (Fig. 6).

Table 2 Mortality and oxidative stress state of zebrafish embryos after the treatments of $CdSO_4$-ANF mixtures and $CdSO_4$

Treatment	Morality (%)	GSH level (%)	SOD activity (%)	MDA content (%)
Simultaneous addition of $CdSO_4$ and ANF (4–24 hpf)[1]	31.11±6.12	62.32±9.48	33.55±14.26	161.75±44.57
Addition of ANF 12 h after the treatment of $CdSO_4$ alone (16–36 hpf)	15.23±2.04[a]	75.51±8.51[a,b]	78.03±5.34[a,b]	122.57±25.45
$CdSO_4$ only (4–36 hpf)	3.96±4.19	101.12±12.14	94.84±10.07	107.14±20.53

Biomarkers are measured after 20 h treatment. Data, which are relative values to the control, are expressed as mean±SD of three independent experiments. [1] Results were obtained directly from Figs. 3 and 5. [a] $P<0.05$, compared with the groups treated by the simultaneous addition of $CdSO_4$ and ANF; [b] $P<0.05$, compared with the groups treated by $CdSO_4$ only

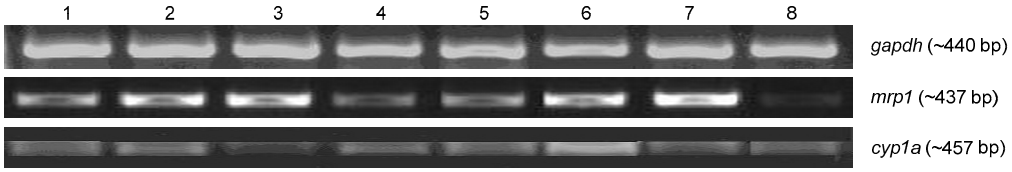

Fig. 6 RT-PCR analysis of the gene expression of *gapdh*, *cyp1a*, and *mrp1*

Results by PCR were from zebrafish embryos at 24 hpf (Lanes 1–4) and 48 hpf (Lanes 5–8). Lanes 1&5: untreated embryos; Lanes 2&6: embryos treated by 75 mg/L $CdSO_4$; Lanes 3&7: embryos treated by 1.5 mg/L ANF; Lanes 4&8: embryos treated by mixtures of 75 mg/L $CdSO_4$ and 1.5 mg/L ANF

RT-PCR results revealed that at sub-lethal concentrations, both $CdSO_4$ and ANF could significantly induce the expression of *mrp1*, correlating well with the previous reports and indicating that Mrp1 was involved in the detoxification of $CdSO_4$ and ANF (Long *et al.*, 2011c; Costa *et al.*, 2012; Navarro *et al.*, 2012). Based on the previous experiments, PAH and heavy metals can act as both the substrates and the competitive inhibitors of Mrps, and thus increase the tissue accumulation of each other when they were used together, which should be the reason for the enhanced toxicity of $CdSO_4$-ANF mixtures. In addition, the reduced gene expression of *mrp1* by the co-treatment of $CdSO_4$ and ANF could be explained by the fact that such $CdSO_4$-ANF mixtures exceeded the capability of ABC transporters and caused significant death of zebrafish embryos (Fig. 4), thus decreasing the mRNA level of *mrp1* and further enhancing the toxicity of $CdSO_4$-ANF mixtures. However, research on the role of multixenobiotic resistance (MXR) in the interaction of environmental chemicals is still rare, and further investigations are needed.

Furthermore, gene expression of *cyp1a* in zebrafish embryos at 24 hpf was found to be significantly enhanced by ANF, but the addition of $CdSO_4$ severely down-regulated the mRNA expression level of *cyp1a*. Furthermore, the co-treatment of $CdSO_4$ and ANF decreased the gene expression to a level near to the control group. In previous experiments, the induction of *cyp1a* by various PAH like benzo[α]pyrene and phenanthrene has been revealed to be a protective mechanism of the cardiovascular dysfunction in the development of zebrafish embryos (Billiard *et al.*, 2008; Wills *et al.*, 2009). Thus, the decreased gene expression of *cyp1a* by $CdSO_4$ could significantly enhance the toxicity of ANF, especially when 48 hpf cardiac edema was selected as the representing endpoint for ANF.

It needs to be mentioned that, cadmium and PAH levels in the rivers are 0.01–0.19 mg/L (Singh *et al.*, 2008) and 0.2–2.0 μg/L (Zhang *et al.*, 2004), respectively, which are much lower than the concentrations used in this experiment. Such high concentrations could be first explained by the bioaccumulation of pollutants in the fish, making the tissue concentration many times higher than that in the surrounding waters (Vergauwen *et al.*, 2013). Secondly, high concentrations of toxicants were usually used in laboratories to determine the possible toxic mechanism since they could exhibit significant effects in the short-term (Shi *et al.*, 2008; Yu *et al.*, 2012). In previous experiments, Konishi *et al*. (2006) found that the 72 hpf LC_{50} (lethal concentration 50%) for $CdCl_2$ was 1000 μmol/L (≈208 mg/L $CdSO_4$), which was similar to the concentration used in our experiments. ANF caused no death of embryos even to its solubility limit of 3 mg/L (data not shown) in this experiment, which should be due to its low oxidative damage (Fig. 4) and induced gene expression of *mrp1* and *cyp1a* (Fig. 6). This result corresponded well with the previous reports where 10 μmol/L ANF (2.72 mg/L) caused no death in differential fishes and was only used as CYP inhibitors (Meinelt *et al.*, 2001; Koenig *et al.*, 2012).

5 Conclusions

In conclusion, an enhancement of developmental toxicity by each other was observed when $CdSO_4$ and ANF were used together. Production of oxidative stress and altered expression of *mrp1* and *cyp1a* could be the important components of such joint toxicity. In the future, this result should be confirmed with more typical PAH, and the involvement of MXR in the interactions of $CdSO_4$ and ANF should be investigated in detail.

Compliance with ethics guidelines

Jian YIN, Jian-ming YANG, Feng ZHANG, Peng MIAO, Ying LIN, and Ming-li CHEN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要：

本文题目：硫酸镉与 α-萘黄酮对斑马鱼发育的个体毒性与联合毒性研究

Individual and joint toxic effects of cadmium sulfate and α-naphthoflavone on the development of zebrafish embryo

研究目的：研究硫酸镉与 α-萘黄酮对斑马鱼发育的个体毒性与联合毒性，并探讨其中的联合毒性机制。

创新要点：首次对硫酸镉与 α-萘黄酮的联合毒性进行了研究，并探索了氧化应激、ATP 结合盒式（ABC）转运蛋白及细胞色素 P450 蛋白（CYP）1A 在化合物联合毒性中的作用。

研究方法：在不同时间点，依据形态学指标检测硫酸镉与 α-萘黄酮单独使用与联合使用时对斑马鱼胚胎的致死与致畸性毒性。取样品，采用试剂盒检测不同处理状态下斑马鱼胚胎中谷胱甘肽（GSH）、超氧化物歧化酶（SOD）及丙二醛（MDA）三个氧化还原指标的改变，聚合酶链式反应（PCR）检测样品中多药耐药蛋白（*mrp*）*1* 及 *cyp1a* 基因表达水平的改变。

重要结论：硫酸镉与 α-萘黄酮的联用能够产生远大于个体且显著增强各自的毒性，而这种增强作用机理在于氧化应激压力的产生，以及两者合用下 *mrp1* 及 *cyp1a* 基因表达水平的改变。

关键词组：联合毒性；硫酸镉；α-萘黄酮；斑马鱼胚胎；氧化应激压力