



# Benomyl-induced development and cardiac toxicity in zebrafish embryos

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## Abstract

Benomyl is a highly effective broad-spectrum fungicide widely used worldwide to control vegetable, fruit, and oil crop diseases. However, the mechanism of its toxicity to aquatic organisms and humans remains unknown. In this study, zebrafish were used to determine the toxicity of benomyl. It was found to be highly toxic, with a 72-h post-fertilization (hpf) lethal concentration 50 (LC<sub>50</sub>) of 1.454 mg/L. Benomyl induced severe developmental toxicity, including shorter body length, slower heart rate, and a reduced yolk absorption rate. Benomyl also increased oxidative stress in zebrafish, especially in the heart and head, as well as increasing malondialdehyde (MDA) content and decreasing catalase (CAT) and superoxide dismutase (SOD) activities. This indicates that benomyl induced reactive oxygen species (ROS) production and cell membrane peroxidation *in vivo*. Acridine orange (AO) staining and apoptosis factor detection further indicated that benomyl induced apoptosis in zebrafish. Overall, these findings demonstrate that benomyl disrupts cellular homeostasis by activating oxidative stress in zebrafish, resulting in an imbalance of cardiac development-related gene expression and apoptosis, which causes severe developmental toxicity and cardiac dysfunction. This study evaluated the *in vivo* toxicity of benomyl, which is a potential threat to aquatic organisms and humans. Possible toxicity mechanisms are explored, providing a valuable reference for the safe use of benomyl.

**Keywords** Benomyl · Zebrafish · Cardiotoxicity · Oxidative stress · Apoptosis

## Abbreviations

hpf	Hours post-fertilization
ROS	Reactive oxygen species
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
AO	Acridine orange

## Introduction

Benomyl is a highly effective and broad-spectrum endosorbent fungicide for spraying, seed dressing, and soil treatment. It is mainly used to control diseases of vegetables, fruits, and oil crops. In 1988, 1,700 tons of benomyl were used on more than 70 crops in 50 countries (Min and Kang 2008). Benomyl effectively kills pathogens by inhibiting the formation of spindles during cell division (Kim et al. 2009). Benomyl has recently been banned in the USA and Europe due to its toxic effects on the environment (Alejandro et al. 2021); however, it is still widely used and has been identified as a contaminant in agricultural products in many developing countries (EFSA et al. 2020). If used frequently and in large quantities, benomyl may saturate the soil and contaminate groundwater or surface water close to the application site. Benomyl has been found at concentrations of 2.6 µg/L in water and 6 µg/kg in the muscles of Nile tilapia from Tenango dam, Puebla, Mexico, and the bioconcentration factor (BCF) revealed that the fungicide was potentially bioaccumulable in the muscle across all collecting seasons (Alejandro et al. 2021).

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Furthermore, carbendazim, a metabolite of benomyl, ranked first in the detection (77.1%) and maximum concentration (4,516 ng/g) of pesticide residues in agricultural products in mainland China (Dai et al. 2021).

In the early 1970s, after more than a decade of use, the toxic effects of benomyl began to be realized. Undegraded benomyl and its decomposition products were found in mung beans, demonstrating that benomyl was not metabolized in plant tissues as rapidly as previously thought (Jhooty and Singh 1972). The half maximum effective concentration ( $EC_{50}$ ) and lethal concentration 50 ( $LC_{50}$ ) values of benomyl were further estimated based on the toxicity effects on four freshwater organisms (*Chlorella*, *Daphnia*, *Lebistes*, and *Salmo*) (Canton 1976). Subsequent mammalian studies have shown that both oral and intraperitoneal benomyl injections show route-independent effects on hepatic microsomal mixed-function oxidases (MFOs) activity and are not toxic to the liver (Dalvi 1992). In Nile tilapia, the in vivo hepatotoxicity of benomyl may, in part, result from changes in hematological parameters, and antioxidants may provide limited protection against benomyl toxicity (Min and Kang 2008). In terms of reproductive toxicity, benomyl induced brain aromatase and toxic effects on zebrafish embryos, but the specific mechanisms of this toxicity are unknown (Kim et al. 2009). In addition, studies on the mechanism of benomyl toxicity in rat cardiomyocyte (H9c2) cells showed oxidative stress-related DNA damage and apoptosis (Mehtap et al. 2021). There are currently no in-depth studies on the cardiotoxicity of benomyl in vivo; therefore, more in vivo toxicological studies are necessary for this widely used fungicide.

As a model animal, zebrafish has numerous advantages, such as low aquaculture cost, short reproductive cycle, and high embryo yield. This enables many environmental pollutants to be screened and their toxicity assessed on a large scale. Furthermore, zebrafish have transparent embryos, so during in vitro fertilization, the damage to ontogeny, organ function, and tissue structure after pollutant exposure can be visually observed (d'Amora et al. 2022; Lin et al. 2011; Pereira et al. 2019). In addition, the zebrafish genome is up to 87% identical to the human genome, and many human disease-related genes have high homology with zebrafish (Wang et al. 2021). Therefore, the use of zebrafish in toxicology, developmental biology, drug screening, and molecular mechanism research of major human diseases is rapidly increasing (Bhagat et al. 2020b; Choi et al. 2021; Gut et al. 2017; Rothenbücher et al. 2019). In this paper, zebrafish were used to assess the toxic effects of benomyl, focusing on cardiotoxicity.

The zebrafish heart, composed of ventricles and atria, is the first organ to function during embryonic development and is highly similar to the human heart in terms of morphology, physiology, gene homology, and regulatory pathways

of gene expression (Bournele and Beis 2016; Huang et al. 2020b; Ma et al. 2021; Thisse and Zon 2002). The zebrafish embryonic heart develops rapidly, and cardiac function is easily observed and quantified. The zebrafish larval compartment at 3-h post-fertilization (hpf) is similar to that of the 3-week human embryonic heart (Epstein and Epstein 2005). Previous related studies have shown that benomyl may induce reproductive and cardiotoxicity in zebrafish, but the pathogenesis is unknown. In this study, zebrafish were used to assess the development and cardiac toxicity of benomyl, and the cardiotoxicity mechanism of benomyl was explored by phenotypic analysis of cardiotoxicity, determining the oxidative stress and apoptosis response, and detection of key marker genes of heart development in zebrafish. The results can provide more evidence for the scientific and rational use of benomyl.

## Materials and methods

### Chemicals and reagents

Benomyl pesticide (50%) was purchased from Lanfeng Biochemical Co. Ltd. (Jiangsu, China). This study is aimed at evaluating the effect of benomyl fungicide on aquatic organisms in the environment, and 50% wettable powder was the most commonly used benomyl pesticide; therefore, the powder was used in the experiment. Acridine orange (CAS: 10127-02-3) was purchased from Solarbio Life Sciences Co. Ltd. (Beijing, China). RNA extraction and reverse transcription kits were purchased from TransGen Biotech Co. Ltd. (Beijing, China). Malondialdehyde (MDA), reactive oxygen species (ROS) reagents, and enzyme assay kit (e.g., superoxide dismutase [SOD] and catalase [CAT]) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents of analytical grade were purchased from Sangon Biotech Co. Ltd. (Shanghai, China).

### Zebrafish husbandry

Wild-type (AB strain) zebrafish were used for total RNA extraction and enzyme activity detection; transgenic lines Tg (*myl7: GFP*) were used for cardiotoxicity phenotype analysis; Tg (*myl7: GFP; kdrl: mCherry*) were used for cardiovascular phenotypic analysis. Zebrafish were purchased from the China Zebrafish Resource Center and maintained in a 14-h:10-h light/dark cycle with a temperature of  $28 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$  (Lu et al. 2013). Male and female zebrafish were isolated overnight in spawning boxes at a ratio of 1:1 or 2:1. Egg-laying was induced under light after baffle removal the next morning (Xiong et al. 2019). Embryos were collected with sterile Petri dishes within 1 hpf and cultured in an incubator at  $28 \text{ }^{\circ}\text{C}$  (Yuan et al.

2021b). Finally, healthy embryos at the epiboly stage were selected at 5.5–6 hpf for subsequent exposure experiments (Kimmel et al. 1995).

## Benomyl exposure

The stock solution of benomyl (1.2 mg/mL) was dissolved in dimethyl sulfoxide (DMSO). At 6 hpf, viable embryos were transferred to 6-well plates, with approximately 20 embryos per well. Benomyl exposure was started from 6 h until 72 h. Exposure solutions were entirely replaced daily. Survival curves counted every 24 h were obtained from acute exposure experiments at five concentrations (0, 0.5, 1.0, 1.5, and 2 mg/L). In addition, ten concentrations were used to fit the dose–response curve (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, and 4 mg/L) and calculate  $LC_{50}$  at 72 hpf. Based on the phenotypes observed in previous experiments (no significant toxicity to zebrafish embryos at <0.75 mg/L and a large number of abnormal embryos at > 1.25 mg/L), three concentrations were selected for the subsequent exposure experiments: 0.8, 1.0, and 1.2 mg/L, while the embryo culture medium with 0.01% DMSO as a control. For the fluorescence phenotype analysis, 0.003% 1-phenyl-2-thiourea (PTU) was used instead of the embryo culture medium to reduce melanin production.

## Morphological quantification

A quantitative analysis of the morphology and function of the zebrafish heart was performed according to (Zhong et al. 2021). Heart rate (beats per minute) and mortality were calculated under an optical microscope (Leica S9i, Germany) at 72 hpf. Body length, yolk area, and pericardial edema area were captured by a stereomicroscope (Leica M205 FA, Germany) and calculated using Image-J software. Cardiovascular morphology was photographed with a confocal microscope (Leica TCS SP-8, Germany). The number of statistics in each group should not be less than two-thirds of the total number of treatments, as shown in Fig. 2A.

## Histology

After paraformaldehyde fixation, tissue sections from each group at 72 hpf were dehydrated in an ascending ethanol series and embedded in paraffin wax blocks (Huang et al. 2020a). Hematoxylin and eosin (HE) staining was employed to evaluate the cardiac morphogenesis of the sections. Subsequently, the stained slices were sealed with neutral resin (Fischer et al. 2005). A microscope (Leica DM2500, Germany) was used to observe histopathological changes.

## Gene expression analysis

Total RNA was extracted from 30 embryos from each group (three treatments and the control) at 72 hpf. RNA extraction and reverse transcription reactions were carried out according to the manufacturer's instructions (Cao et al. 2019). Quantitative real-time polymerase chain reaction (qPCR) was used to detect gene expression related to cardiac development (three replicates per group).  $\beta$ -Actin was used as an internal reference gene. The primers for this study were taken from previous publications (Cao et al. 2019; Huang et al. 2018; Meng et al. 2020; Wei et al. 2021). Each experiment was performed in triplicate.

## Oxidative stress analysis

ROS, SOD, and CAT activity and malondialdehyde (MDA) content were measured to determine the effects of benomyl exposure on oxidative and antioxidant levels in zebrafish larvae (Huang et al. 2020a). For ROS fluorescence measurements, live zebrafish larvae at 72 hpf were incubated in oxidant sensitive fluorescent probe (20  $\mu$ M DCFH-DA) at 28 °C in the dark for 30 min (Huang et al. 2020a; Jin et al. 2020; Wang et al. 2019a). The excess probe was removed by embryo culture medium. ROS production was determined by fluorescence intensities, recorded by a fluorescence stereomicroscope (Leica M165 FA, Germany) at a constant setting. In addition, SOD and CAT activity and MDA content were measured by a PerkinElmer multifunctional micrometer (Huang et al. 2020a). Fifty embryos were collected per treatment in triplicate.

## Apoptosis detection

The degree of apoptosis induced by benomyl exposure was determined by AO staining (Tucker and Lardelli 2007). Larvae were washed with an embryo culture medium three times to remove benomyl residues at 72 hpf, followed by incubation with 5 mg/L AO working solution at 28.5 °C in the dark for 30 min (Cheng et al. 2020; Jin et al. 2020). Photographs were taken with a fluorescence stereomicroscope (ZESIS Zoom. V16, Germany). Apoptosis-related genes were also detected.

## Statistical analysis

GraphPad Prism 8.3.0 (San Diego, CA, USA) software was used for statistical analysis. Significant differences were determined using one-way analysis of variance (ANOVA) followed by Dunnett's test. All results are presented as mean  $\pm$  standard deviation (sd), and *p* values less than 0.05, 0.01, or 0.001 were considered statistically significant (0.05) or extremely significant (0.01, 0.001) (Cao et al. 2020).

The lethal concentration 50 (LC<sub>50</sub>) was obtained by simple logistic regression analysis in Origin Pro 8.5 software (OriginLab Corporation, Northampton, MA, USA).

## Results

### Morphological observations and acute toxicity of benomyl exposure

The molecular formula of benomyl carbendazim is shown in Fig. 1A. To determine its toxicity to aquatic organisms, zebrafish embryos were exposed to different concentrations of benomyl, and the cumulative survival rates were calculated at different time points. Benomyl caused high mortality of embryos in a concentration-dependent manner (Fig. 1B). Based on the survival rate of the tested concentration and the LC<sub>50</sub> at 72 hpf (Fig. 1C), benomyl at 0.8, 1.0, and 1.2 mg/L were used for the final experiments.

Body length gradually decreased at concentrations of 1.0 and 1.2 mg/L (Fig. 1D), and the yolk sac area gradually increased (Fig. 2D). In addition, severe spinal curvature began to appear at 1.2 mg/L (Fig. 2A). These results indicate that benomyl induces concentration-dependent developmental toxicity on zebrafish embryos.

### Cardiotoxicity caused by benomyl exposure

The heart is one of the earliest organs to function during zebrafish ontogeny. Morphological observations revealed that the sizes of atria and ventricles were significantly reduced after benomyl exposure, and linear stretching increased the distance between the atria and ventricles (Fig. 2A). In addition, heart rate decreased gradually in a concentration-dependent manner, with severe pericardial edema (Fig. 2B, C). HE staining showed that the number of blood cells in the atria and ventricles decreased significantly (Fig. 3). Confocal laser scanning showed cardiovascular abnormalities, with a decrease in cardiovascular number and

growth disorders after benomyl exposure (Fig. 4), indicating that benomyl exposure causes severe cardiotoxicity.

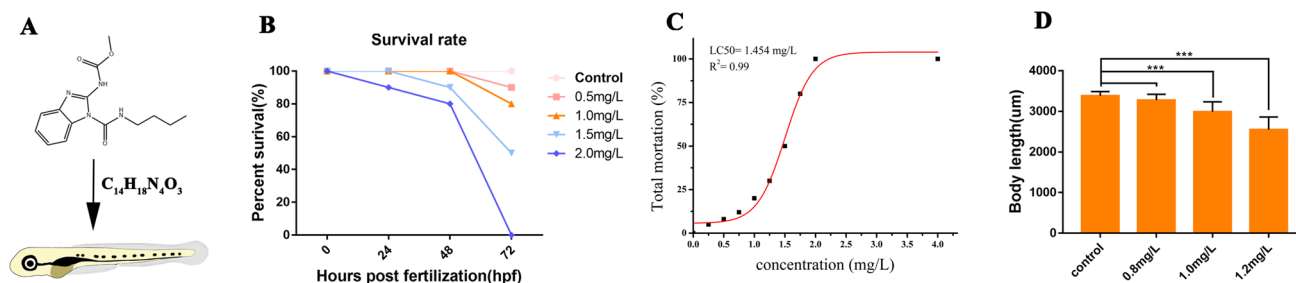
### Benomyl exposure affects heart development-related gene expression levels

The above experiments show that benomyl causes cardiotoxicity in zebrafish; therefore, the expression of genes related to heart development was analyzed further. Significant differences in the expression of genes related to heart development were identified between the control and treatment groups: *nppa* was upregulated in a concentration-dependent manner, *vmhc* and *tbx2b* were downregulated in a concentration-dependent manner, and other genes (*myh6*, *nkx2.5*, *gata4*) showed varying degrees of downregulation (Fig. 5), indicating that benomyl exposure alters the expression of key heart development genes.

### Benomyl exposure increases oxidative stress

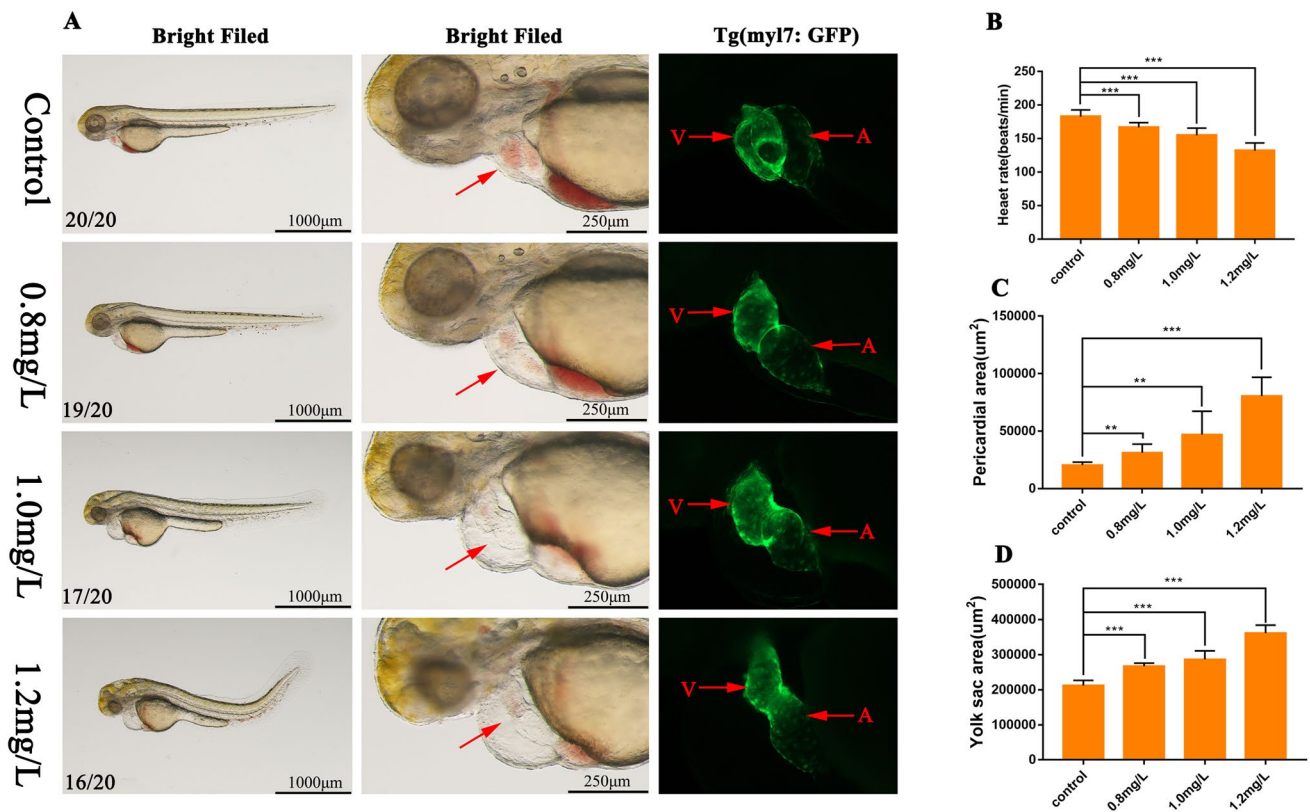
ROS formation is a natural by-product of normal oxygen metabolism and plays an important role in cell signaling and homeostasis. However, ROS levels may increase significantly in response to certain environmental stresses or external drug stimuli, causing severe damage to cellular structures, such as cellular proteins, nucleic acids, and the cytomembrane, eventually triggering a cascade of apoptosis. Endogenous ROS are normally removed by antioxidant enzymes such as SOD and CAT, and MDA is a commonly used index of membrane lipid peroxidation.

Following benomyl exposure, oxidative stress increased significantly. ROS fluorescence intensity also increased with benomyl concentration (Fig. 6A, B), while benomyl exposure led to a dose-dependent increase in MDA (Fig. 6C) and decreased CAT activity (Fig. 6E). SOD activity first decreased and then increased slightly (Fig. 6D). These results indicate that oxidative damage is an important cause of cardiotoxicity in zebrafish after benomyl exposure.



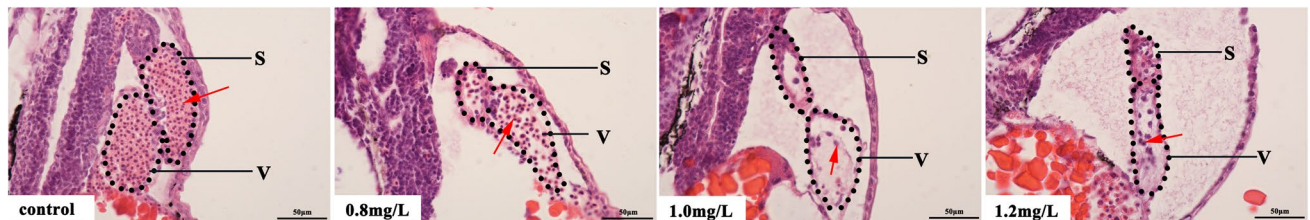
**Fig. 1** Developmental toxicity of benomyl in zebrafish embryos. **A** The benomyl formula for toxicity testing in zebrafish. **B** Survival rate of embryos at 24, 48, and 72 h post-fertilization (hpf). **C**

Dose–response curve at 72 h after benomyl exposure. **D** Body length of larvae at 72 hpf. Data are represented as mean  $\pm$  SD (\* $p$  < 0.05, \*\* $p$  < 0.01, and \*\*\* $p$  < 0.001)



**Fig. 2** Effects of benomyl exposure of zebrafish embryos at 72 h post-fertilization (hpf). **A** Typical bright field images and an enlarged fluorescence microscopy view of the heart. **B** Effects of different con-

centrations of benomyl on the heart rate of zebrafish embryos. **C** Pericardial area (um<sup>2</sup>). **D** Yolk sac area (um<sup>2</sup>). A, atrium; V, ventricle. Data represent means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$



**Fig. 3** Hematoxylin and eosin staining of heart sections at different concentrations of benomyl-exposure at 72 h post-fertilization (hpf) in zebrafish larvae. A, atrium; V, ventricle. The red arrow shows the red blood cells inside the heart. Scale = 50 um

### Benomyl exposure increased apoptosis in zebrafish larvae

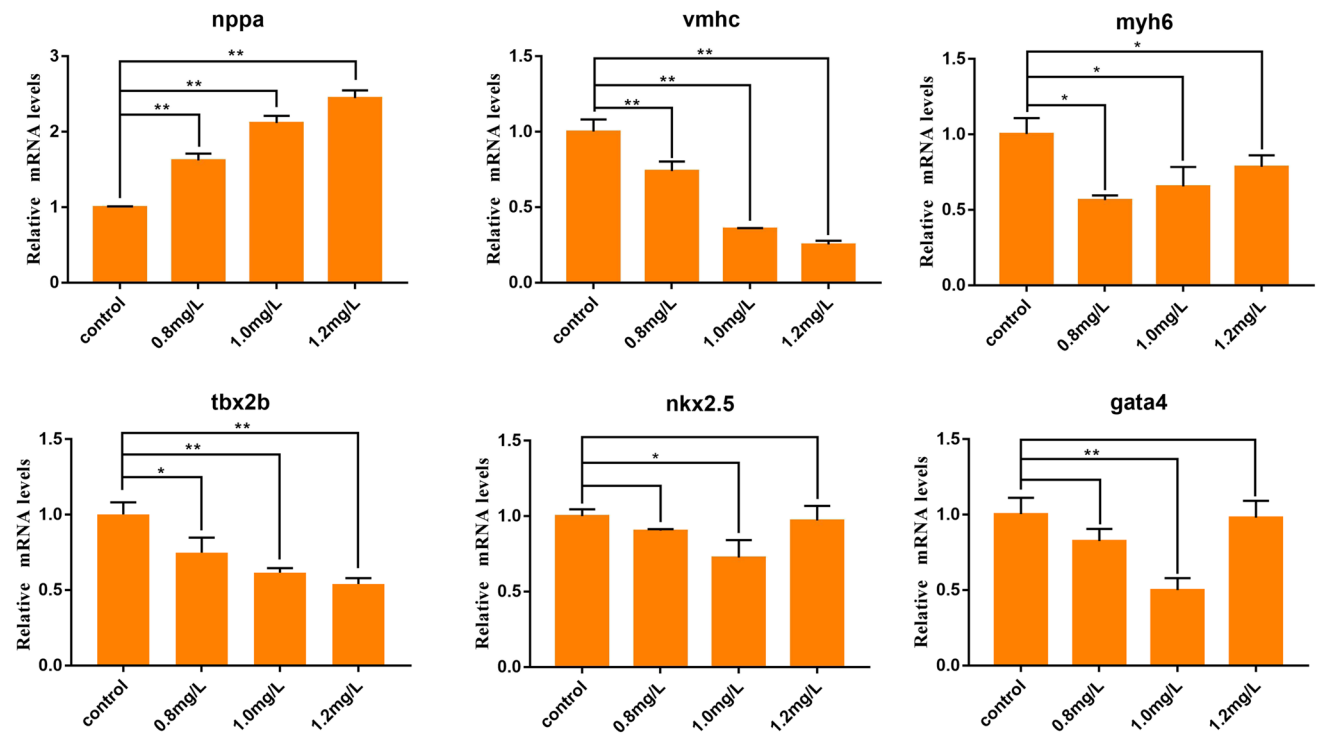
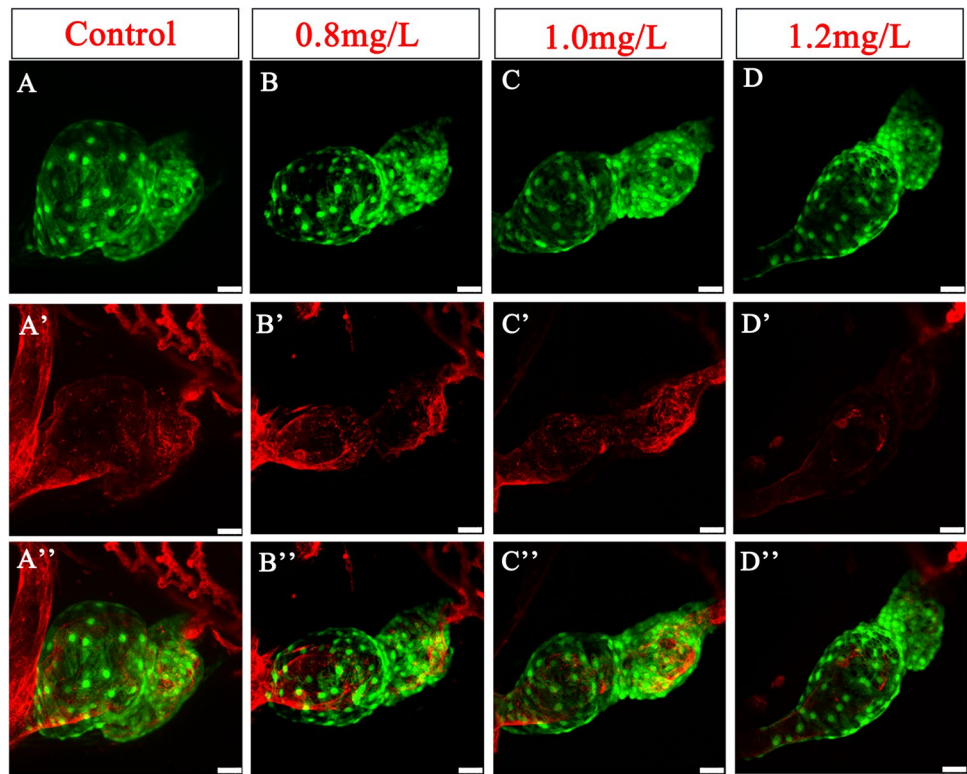
Excessive oxidative stress can cause DNA damage and ultimately induce apoptosis. To investigate the effect of benomyl on apoptosis in zebrafish larvae, larvae were stained with AO reagent at 72 hpf and found that apoptosis increased significantly after benomyl exposure, mainly in the head and heart (Fig. 7A). Furthermore, the expression levels of apoptosis-related genes were determined and found that *caspase9*, *caspase3*, and *p53* were significantly upregulated in the treatment group (Fig. 7B-F), and *bcl2*

was downregulated in a concentration gradient (Fig. 7C). In conclusion, benomyl exposure induces apoptosis in zebrafish larvae.

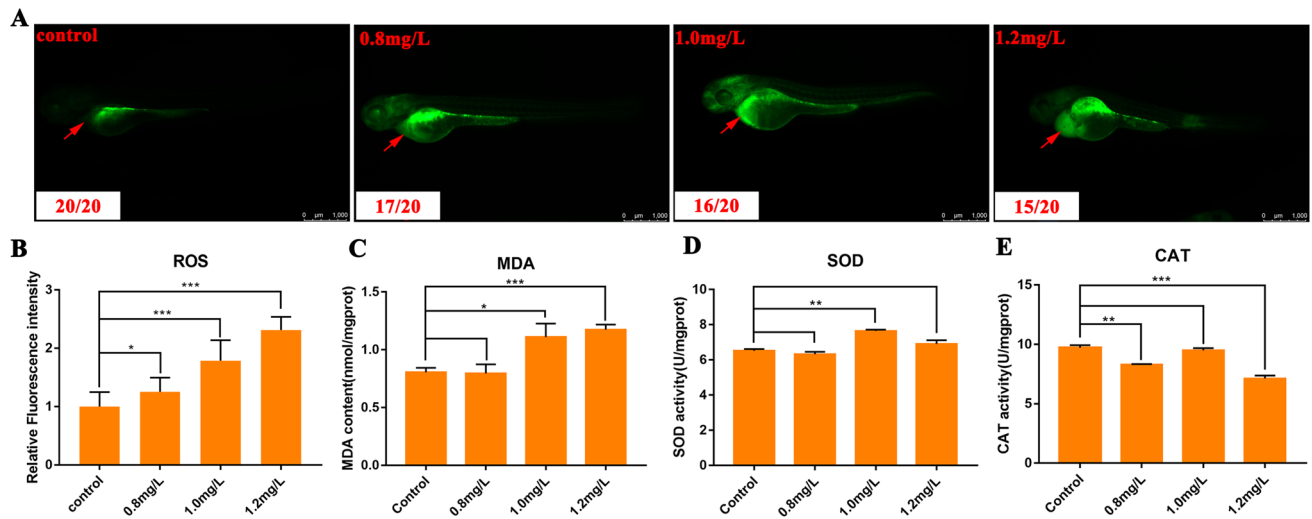
### Discussion

Benomyl is a highly effective, broad-spectrum endosorbent fungicide with protective, eradicating, and long-term therapeutic effects. It can effectively control various crop diseases caused by ascomycetes, basidiomycetes, and semi-anemones. Benomyl has recently been banned in some

**Fig. 4** Exposure to benomyl leads to dysplasia of the cardiovascular system in zebrafish larvae at 72 h post-fertilization (hpf). Confocal microscopy images of the heart using Tg (*myl7: GFP; kdr1: mCherry*) transgenic lines. The green fluorescence represents the heart, and the red fluorescence represents the cardiovascular system. **A–D** The atria and ventricles; **A’–D’** the cardiovascular system; **A’’–D’’** merge. Scale = 50  $\mu$ m

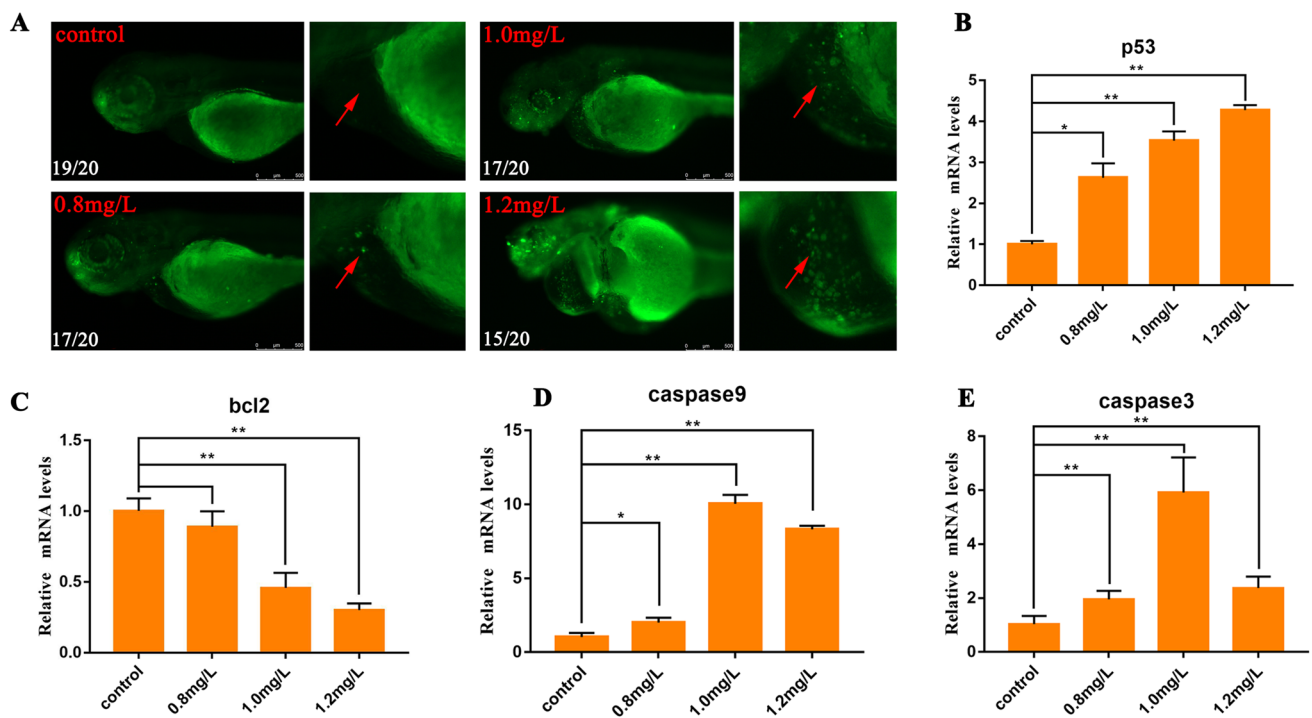


**Fig. 5** Expression level of heart development-related genes in zebrafish larvae after exposure to benomyl. Data represent means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$



**Fig. 6** Benomyl-induced oxidative stress in zebrafish larvae. **A** Distribution of fluorescence visualizing of reactive oxygen species (ROS) in zebrafish larvae after exposure to benomyl at different concentrations. **B** Statistics of the relative fluorescence intensity of the image in

**A, C–E** Concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT). The data represent means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$



**Fig. 7** Apoptosis induced by benomyl in zebrafish larvae at 72 h post-fertilization (hpf). **A** Acridine orange (AO) staining of zebrafish embryos at 72 hpf following exposure to different benomyl concentra-

tions; **(B–E)** Expression levels of apoptosis-related genes. Data represent means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$

developed countries due to its environmental effects, but it is still widely used in many developing countries and is identified as a contaminant in agricultural products (EFSA et al. 2020). Therefore, in this study, zebrafish were used

to evaluate the potential toxicity of benomyl. The findings showed that benomyl leads to serious developmental toxicity and cardiotoxicity in zebrafish larvae. With the increase in exposure concentration, the survival rate gradually decreases

and changes the expression levels of key cardiac development genes. The main developmental effects were a reduction in body length and an increase in yolk area, suggesting that exposure to benomyl at early stages could affect spine development and yolk uptake rates. While cardiotoxic effects included decreased heart rate, atrial ventricular ring failure leading to linear elongation, reduced cardiomyocytes, and severe deterioration of pumping function, which may be closely related to congenital heart disease, indicating that the developing heart is possibly a previous target of benomyl.

Many genes play an important regulatory role in the zebrafish heart development process, and the cooperation of these genes ensures the normal functioning of the heart (Charli et al. 2016; Huang et al. 2020b). *Nppa* is an early specific marker of the heart that distinguishes the developing atria from the working myocardium of ventricles. Ventricular *nppa* expression is downregulated around birth and reactivated in response to various cardiovascular diseases (Houweling et al. 2005). In this study, the expression of *nppa* increased with benomyl concentration in zebrafish embryos, implying that benomyl may cause cardiovascular disease. *Vmhc* is one of the early cardiac development marker genes, initially expressed in the anterolateral mesoderm of the embryo and thereafter restricted to cardiomyocytes in the ventricles (Han et al. 2015). *Myh6* is a key transcription factor that promotes ventricular cardiomyocyte expansion, which is expressed in the myocardium; it is closely related to atrial septal defect and cardiac conductive disease (Ma and Li 2021; Yuan et al. 2021a). In this study, both *vmhc* and *myh6* were downregulated by benomyl, indicating that the expression of these two genes was inhibited by benomyl exposure. This could lead to congenital heart disease, heart conduction disease, and hyaline body myopathy. Misexpression of *tbx2b* in zebrafish myocardium can block atrioventricular canal formation and cardiac looping (Chi et al. 2008). *Nkx2.5* plays a crucial role in the differentiation and maturation of cardiomyocytes and regulates atrioventricular formation (Sultana et al. 2008; Wang et al. 2019c). Previous studies have found significant defects in the ventricles and atria of *nkx2.5*<sup>-/-</sup> embryos (Harrington et al. 2017). In this study, the expression of *tbx2b* and *nkx2.5* was clearly suppressed in zebrafish embryos after benomyl exposure, which may lead to blocked differentiation of myocardial cells, incomplete development of atria and ventricles, atrioventricular tube formation disorder, and blocked blood circulation. As an important transcription factor, *gata4* regulates the atrial, ventricular, and atrioventricular valvular formation and ensures normal heart development (Gruber 2005). Mutation of *gata4* can lead to heart defects, which is strongly associated with congenital heart disease (Holtzinger and Evans 2005; Zhang et al. 2017); after heart injury in zebrafish, *gata4* expression is triggered to reactivate myocardial cell proliferation, which plays an important

role in cardiac regeneration (Kikuchi et al. 2010). According to these results, benomyl exposure downregulates *gata4*, which may be an important cause of cardiac dysplasia. All these genes play a key role in early heart development: normal heart development results from their cooperation, and the dysregulation of the expression of any gene may cause cardiac dysplasia.

Environmental chemicals, clinical medication, and some physical factors can lead to cardiotoxicity (Bhansali et al. 2019; Mamoshina et al. 2021). Although some studies have shown that RNA-binding protein QKI (Quaking) and neutrophils play a role in heart disease (Bernstein 2018; Bhagat et al. 2020a), however, oxidative stress is still a major cause of cardiotoxicity, and ROS plays a key role in the damage and pathogenesis of cardiac tissue (Ping et al. 2020).

Harmful environmental stimuli can cause an oxidative stress response in organisms, resulting in the accumulation of ROS, DNA damage, and ultimately cell apoptosis. (Huang et al. 2018; Wang et al. 2019a, b). In this study, oxidative stress in zebrafish embryos increased in a gradient with increasing benomyl concentrations, suggesting an imbalance between oxidation and antioxidation in vivo. SOD is an important antioxidant enzyme widely distributed in the body; its activity level can indirectly reflect the oxidative stress level in the body. CAT activity and MDA content are also important oxidative stress markers (Karadeniz et al. 2011; Monteiro et al. 2006). Therefore, the activity of SOD and CAT, as well as the MDA content, were analyzed, and the lipid oxidation product MDA was found to increase in a dose-dependent manner gradually. SOD activity decreased first, then increased slightly, and CAT activity decreased with increasing concentrations of benomyl, suggesting that benomyl exposure could increase oxidative stress, leading to lipid peroxidation and depletion of intracellular related antioxidant enzymes. Inhibition of SOD enzyme activity may induce mitochondrial oxidative stress and cardiomyocyte hypertrophy (Dubois-Deruy et al. 2017), hearts exhibiting ventricular hypertrophy, and increased interstitial fibrosis may be related to decreased CAT enzyme activity caused by a low-protein diet (Ferreira et al. 2022). The decreased SOD and CAT enzyme activities are closely related to the development of cardiac toxicity. Previous studies have also shown that benomyl exposure can lead to glutathione depletion in rats (Banks and Soliman 1997), which is consistent with the results of this study. In addition, oxidative stress-induced aging has been proven to be one of the major factors contributing to the increased incidence of cardiovascular and cerebrovascular diseases (Izzo et al. 2021). Reactive oxygen species can change the structure of cardiomyocyte organelles through oxidative damage to a variety of biological macromolecules, thereby leading to systolic dysfunction (Dubois-Deruy et al. 2017), and excessive ROS produced will lead to irreversible damage mitochondria, become the important



factor in the development of cardiovascular disease (Bhatti et al. 2017). These results suggest that the cardiotoxicity resulting from benomyl exposure is likely caused by oxidative stress-induced cardiomyocyte injury and apoptosis, ultimately leading to severe cardiac defects. In addition, cardiovascular development was significantly inhibited, and with increasing benomyl concentrations, the cardiovascular number was significantly reduced, and the distribution was irregular.

Apoptosis is a basic cellular process with an important role in removing unwanted or abnormal cells in multicellular organisms (Knabe and Washausen 2022; Lossi 2022). Apoptosis can be divided into exogenous and endogenous apoptosis. The endogenous pathway is activated by the accumulation of intracellular p53, caused by cellular stress, DNA damage, and other factors (Liu et al. 2022). As a typical anti-apoptotic factor, bcl2 is located in the inner membrane of mitochondria. It controls the permeability of the mitochondrial membrane, affecting the release of apoptosis-related factors (such as cytochrome C) and then activating caspase9 and caspase3 (Flores-Romero and García-Sáez 2019). In this study, the expression of apoptosis-related genes p53, caspase9, and caspase3 significantly increased following benomyl exposure, while the expression of bcl2 significantly decreased, which is consistent with previous studies of another benzimidazole fungicide, thiophanate-methyl (Jia et al. 2020). Accordingly, it can be speculated that benomyl activates the endogenous apoptosis cascade in zebrafish larvae via cellular oxidative stress.

## Conclusion

In summary, this work further elucidates the early developmental toxicity of benomyl to zebrafish, focusing on the mechanism of action that causes cardiotoxicity. Benomyl altered the expression of various key genes during zebrafish heart development and reduced the activity of SOD and CAT in vivo by activating oxidative stress and promoting apoptosis, resulting in serious cardiotoxicity. This data provides reference material for the safe use of benomyl.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11356-022-24213-z>.

**Author contribution** Qiang Luo: methodology, investigation, data curation, validation, and original draft writing. Shuqiong Tang: data curation. Xiaoping Xiao: methodology. You Wei: visualization. Bo Cheng: formal analysis. Yong Huang: modifying references. Keyuan Zhong: data curation. Guiyou Tian: modifying references. Huiqiang Lu: conceptualization, supervision, and project administration.

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**Data availability** Data obtained and analyzed in this study are included in this article and available on reasonable request from the corresponding author.

## Declarations

**Ethics approval and consent to participate** All experiments using zebrafish were performed according to the animal protocol and authorized by the laboratory animal welfare and ethics committee of Gannan Normal University (gnnu2022-0628).

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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