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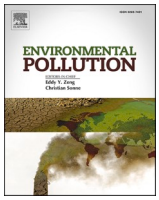

Stereo-selective cardiac toxicity induced by metconazole via oxidative stress and the wnt/β-catenin signaling pathway in zebrafish embryos☆

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ABSTRACT

Metconazole (MEZ), a chiral triazole fungicide, produces enantioselective adverse effects in non-target organisms. Among MEZ's isomers, *cis*-MEZ displays robust antimicrobial properties. Evaluating MEZ and *cis*-MEZ's toxicity may mitigate fungicide usage and safeguard non-target organisms. Our study evaluated the toxicity of MEZ and its *cis*-isomers at concentrations of 0.02, 0.2, 2, and 4 mg L^{-1}. We report stereoselectivity and severe cardiovascular defects in zebrafish, including pericardial oedema, decreased heart rate, increased sinus venous and bulbous arteries distances, intersegmental vessel defects, and altered cardiovascular development genes (*hand2*, *gata4*, *nkx2.5*, *tbx5*, *vmhc*, *amhc*, *dll4*, *vegfaa*, and *vegfc*). Further, MEZ significantly increased oxidative stress and apoptosis in zebrafish, primarily in the cardiac region. Isoquercetin, an antioxidant found in plants, partially mitigates MEZ-induced cardiac defects. Furthermore, MEZ upregulated the Wnt/β-catenin pathway genes (*wnt3*, *β-catenin*, *axin2*, and *gsk-3β*) and β-catenin protein expression. Inhibitor of Wnt Response-1 (IWR-1) rescued MEZ-induced cardiotoxicity. Our findings highlight oxidative stress, altered cardiovascular development genes, and upregulated Wnt/β-catenin signaling as contributors to cardiovascular toxicity in response to MEZ and *cis*-MEZ treatments. Importantly, *1R,5S*-MEZ exhibited greater cardiotoxicity than *1S,5R*-MEZ. Thus, our study provides a comprehensive understanding of *cis*-MEZ's cardiovascular toxicity in aquatic life.

1. Introduction

Metconazole (MEZ) is a well-known chiral triazole fungicide widely utilised for its broad-spectrum effectiveness (Li et al., 2022). MEZ comprises two chiral carbon centres that encapsulate two *cis*- and two *trans*-stereoisomers, with a *cis*-to-*trans* ratio of 85:15 (Álvarez et al., 2023). The focus of current studies primarily centres on the bioactivity and toxicity of MEZ stereoselectivity (Asad et al., 2017), with the two *cis*-MEZ stereoisomers demonstrating superior antibacterial properties compared to their *trans* counterparts (Deng et al., 2021). For instance, *1S, 5R*-MEZ showed 13.9–23.4 times more bioactivity than *1R,5S*-MEZ against *Alternaria triticina* and *Fusarium graminearum Schw* (He et al., 2021). The environmental implications of MEZ are becoming increasingly worrying as it is specifically stereotoxic to aquatic organisms like *Microcystis flos-aquae*, *Daphnia magna*, and *Chlorella proteolytica* (Li et al., 2022; Li et al., 2021; Deng et al., 2019). It is critical to evaluate the potential toxicity of highly antimicrobial isomers in order to reduce the amount of fungicide used and the potential harm to non-target organisms. Hence, in order to fully understand the environmental hazards of MEZ from an enantiomeric perspective, it is imperative to conduct a

Abbreviations: BA, Bulbous arteries; ISQ, Isoquercitrin; ISV, Intersegmental vessel; MEZ, Metconazole; SOD, Superoxide dismutase; SV, Sinus venous; IWR-1, Inhibitor of Wnt Response-1; MDA, Malondialdehyde; NO, Nitrous oxide; ROS, Reactive oxygen species..

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thorough and systematic assessment of the selective toxicity of *cis*-MEZ.

MEZ exhibits stability in water and aquatic environments with half-lives of 1–15 d and 116–814 d, respectively (He et al., 2021), and its water solubility limit is 30.4 mg/L (Álvarez et al., 2023). Residues of MEZ are observed to circulate between soil, water, and sediment. Furthermore, these residues gradually accumulate in aquatic organisms and humans through the food chain, significantly threatening non-target organisms (Deng et al., 2023). *cis*-MEZ accumulates in zebrafish and causes stereoselective acute toxicity (He et al., 2022). The cardiovascular system is prone to harm caused by exogenous agents in its early phases of biological development (Wang et al., 2022b). According to a recent study, *1S,5S*-MEZ is more cardiotoxic in the *trans*-MEZ than *1R, 5R*-MEZ. (Li et al., 2024). Nonetheless, the extent of information about cardiotoxicity from MEZ and its *cis*-isomers at the enantiomeric level is inadequate.

The zebrafish is a widely recognised aquatic model with many qualities that make it an attractive alternative option for basic biomedical research (Glickman and Yelon, 2002; Shen and Zuo, 2020; Tonelli et al., 2020). Despite possessing a single atrium and ventricle, zebrafish exhibit numerous parallels with humans in terms of developmental processes, cardiac function, and responses to pharmacological agents (Yang et al., 2023). Consequently, the pathophysiological alterations in zebrafish cardiac physiology may offer insights into the potential adverse effects of environmental contaminants on human cardiovascular health (Howe et al., 2013; Wang et al., 2023). Recent studies on chiral triazole fungicides have highlighted their ability to induce stereoselective cardiotoxicity in zebrafish (Sun et al., 2020; Perk et al., 2023; Wang et al., 2023). For instance, Jia et al. (2020) demonstrated that penconazole and its enantiomers have the ability to reduce the heart rate in zebrafish embryos, with the (+)-penconazole showing considerably greater toxicity than (−)-penconazole.

Previous studies on triazoles have associated cardiotoxicity with oxidative stress and have implicated that the Wnt pathway may be involved in these effects (Li et al., 2024; Yang et al., 2023). Within the Wnt/β-catenin signaling pathway, the adenomatous polyposis (APC)/axin/glycogen synthase kinase (GSK)-3β-complex and casein kinase 1 quickly phosphorylate and breakdown β-catenin. Disheveled is activated when the Wnt ligand binds to its corresponding receptor, which in turn inhibits GSK-3β. This causes β-catenin to accumulate in the cytoplasm for a longer period of time, which makes it easier for it to engage with transcriptional regulators of the T-cell factor (TCF)/lymphoid enhancer (LEF) family to control the transcription of genes related to cardiovascular health (Bertozzi et al., 2022; Yue et al., 2017). Moreover, a recent study provided compelling evidence of the association between MEZ and cardiotoxicity in zebrafish. The findings demonstrated a notable upregulation of the Wnt pathway through analysis of transcriptomics data (Li et al., 2024). The research on difenoconazole has demonstrated its ability to induce cardiotoxicity in zebrafish, establishing a direct connection between the Wnt pathway, pericardial oedema, and linear stretch (Wang et al., 2023; Yang et al., 2023). Additionally, Othmène et al. (2020) found that a common triazole fungicide named tebuconazole could induce cardiotoxicity in adult rats mainly through cardiomyocyte apoptosis. Wang et al. (2022a) also demonstrated that oxidative stress plays a key role in difenoconazole-induced cardiac damage in carp. In general, oxidative stress and apoptosis has a potentially contribute to the cardiotoxicity induced by these fungicides induce (He et al., 2022; Tait et al., 2022). Recent studies have shown that oxidative stress affects the expression of cardiovascular developmental genes in zebrafish by affecting *rspo1*, an upstream activator of the Wnt/β-catenin signalling pathway (Fu et al., 2022; Yang et al., 2024). Although the accumulation of *cis*-MEZ in zebrafish causes oxidative stress disorders (He et al., 2022), the mechanisms by which MEZ and its *cis*-isomers induce cardiotoxicity through oxidative stress and the Wnt/β-catenin pathway remain to be fully elucidated.

Antioxidants were usually considered effective against cardiotoxicity and diabetes-related vascular complications (Liu et al., 2021). Isoquercitrin (ISQ), a natural antioxidant present in numerous plants (Liu et al., 2023; Bondonno et al., 2020; Melo Branco de Araújo et al., 2013), has been shown to reduce oxidative stress damage caused by Bisphenol A (BPA) in zebrafish (Sahoo et al., 2020). Also ISQ has been shown to attenuate cardiac dysfunction by demonstrating antioxidant stress effects in mice (Huang et al., 2018). Antioxidants are being investigated as possible therapeutic targets for cardiotoxicity (Jiang et al., 2023), however, nothing has been determined about the alleviation of MEZ-induced cardiotoxicity by ISQ and its underlying mechanisms.

Thus, we hypothesised that MEZ and its *cis*-isomers cause different degrees of cardiac damage through oxidative stress and Wnt/β-catenin signaling pathways. In this study, we used genetically modified zebrafish (*myl7:GFP* and *kdrl:EGFP*) to track cardiovascular abnormalities and function. Quantitative real-time PCR (qRT-PCR) and Western blot assays were also performed to identify gene expression and protein level resulting from developmental exposure to MEZ and its *cis*-isomers. Our study aimed to provide new insights into the stereoselective toxicity of MEZ and highlight deficiencies in current research on MEZ.

2. Materials and methods

2.1. Chemicals and materials

MEZ racemate (*rac*-MEZ, purity ≥97%) was purchased from Huifeng Agrochemical Co., Ltd. (Jiangsu, China). *1S,5R*-MEZ and *1R,5S*-MEZ (enantiomeric excess ≥99%) were prepared by Daicel Chiral Technology Co., Ltd. (Shanghai, China). Additionally, the masterbatches used in this study contained 100 mg of each *1S,5R*-MEZ, *1R,5S*-MEZ, and *rac*-MEZ, which were dissolved in 10 mL of dimethyl sulfoxide (DMSO).

2.2. Fish maintenance and embryo collection

This study used wild-type AB and transgenic lines of *Tg(myl7:EGFP)* and *Tg(kdrl:EGFP)* that were purchased from the National Zebrafish Resource Center (Wuhan, China). An aquaculture system (Hunter, Hangzhou, China) maintained zebrafish at a temperature of 28 °C and pH levels ranging from 7.0 to 7.6 with proper water circulation. The fish room was illuminated for 14 h during the day and 10 h in darkness. Two times a day, adult fish were fed live brine (*Artemia salina*). Adult zebrafish at a 1:1 male: female ratio were divided by dividers in spawning tanks for the night before to the egg collecting test. After removing the dividers, fertilised eggs were collected the next morning at 2 h post-fertilisation (hpf). Under a stereomicroscope, fertilised eggs with normal development and no abnormalities were chosen. Zebrafish were treated humanely according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all animal procedures were approved by the Animal Experimentation Ethics Committee of the Chinese Academy of Agricultural Sciences (IACUC2023855301).

2.3. Drug exposure concentrations

Exposure concentrations of *1S,5R*-MEZ, *rac*-MEZ and *1R,5S*-MEZ were established at 0, 0.02, 0.2, 2, and 4 mg L^{-1}. The solvent control (0.04% DMSO) was used as a control group. Each treatment group contained 20 healthy fertilised eggs and 8 mL of the exposure solution and was performed in triplicate. The rescue experiments were treated with 2.5-, 5-, and 10-μM ISQ and 4 mg L^{-1} of *1S,5R*-MEZ, *1R,5S*-MEZ and *rac*-MEZ. To ensure consistent exposure concentrations in the treatment groups, half of the exposure solution volume was replenished with fresh solution every 24 h. Up to 96 hpf, the survival and hatching rates were assessed and computed every 24 h. Recordings of zebrafish embryos and larval morphology were made using a SZX2-FOF microscope (Olympus Corporation, Tokyo, Japan). The embryos' body length and eye area were measured at 96 hpf.

2.4. Histopathological staining

After exposure to 4 mg L^{-1} *1S,5R*-MEZ, *1R,5S*-MEZ, and *rac*-MEZ or control at 96 hpf, the embryos were fixed for 24 h in 4% paraformaldehyde. Zebrafish embryos embedded in paraffin wax were sectioned into 4-μm thick slices and subsequently subjected to hematoxylin and eosin (H&E) staining.

2.5. Assessment of cardiovascular structure and function

Fluorescent imagery of transgenic zebrafish embryos was acquired utilising an SZX16 fluorescence microscope (Olympus Corporation). At 96 hpf, the number of intersegmental vessel (ISV) in *Tg(kdrl:EGFP)* embryos was counted. The morphological alterations of *Tg(myl7:GFP)* embryos were documented by photographs taken at 96 hpf. Several measures, including the pericardial area and the distance between the bulbous arteries (BA) and sinus venous (SV), were computed. Heart rates were assessed for 20 s at 72 and 96 hpf, then multiplied by 3 to determine the heart rate per minute (n = 12).

2.6. Analysis of enzyme activity and reactive oxygen species (ROS) accumulation

After *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ exposure, 30 embryos were taken at 96 hpf from each group. Excess solution was blotted, and the embryos were promptly ground and placed on ice. Superoxide dismutase (SOD) enzymatic activity was measured according to the test kit's manufacturer's instructions (TransGen Biotechnology, Beijing, China), SOD was measured using an enzyme labeller YQ205-01 and the wavelength was adjusted to 560 nm. Three replicates with 30 embryos each were performed for each group.

Twenty embryos were chosen at random from each treatment group. After that, they were left in the dark at 28 °C for 30 min while stained with a 10-μM 2′,7′-Dichlorodihydrofluorescin diacetate (DCFH-DA) probe. After incubation, the embryo was quickly rinsed three times with embryo culture solution. Green fluorescence was detected using an SZX16 fluorescence microscope (Olympus Corporation).

2.7. Acridine orange (AO) staining

The embryos were incubated in 7.0 μg mL^{-1} of acridine orange for 30 min at 28 °C without light after being washed twice with embryo culture fluid at 96 hpf. Following incubation, the embryos were twice rinsed with embryo culture fluid and given tricaine (0.3 g L^{-1}) anaesthesia. Using a SZX16 fluorescent microscope (Olympus Corporation), apoptotic cells in embryos were examined and captured on camera. Twenty embryos were examined per group.

2.8. Western blot assays

For protein expression analysis, the total protein was extracted at 96 hpf from zebrafish embryos treated with 4 mg L^{-1} *1S,5R*-MEZ, *rac*-MEZ and *1R,5S*-MEZ. Equal quantities of protein from each embryo were isolated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to a polyvinylidene fluoride (PVDF) membrane. Following primary antibodies (*β*-catenin antibody, Proteintech, China; Bax, Sanying, China) incubation, the PVDF membranes were treated with secondary antibodies coupled with horseradish peroxidase (HRP). Finally, chemiluminescence was detected using an Amersham Imager 600 (GE) and protein bands analysis was conducted using Image J software.

2.9. Investigation of the gene expression by quantitative RT-PCR (qRT-PCR)

The genes (*hand2*, *gata4*, *nkx2.5*, *tbx5*, *vmhc*, *amhc*, *dll4*, *vegfaa* and *vegfc*) associated with cardiovascular development, wnt/*β*-catenin signaling pathway-related genes (*β-catenin*, *axin2*, *gsk3β*, and *wnt3*), and oxidative stress-related genes (*sod1* and *cat*) were detected by real-time fluorescence quantitative qRT-PCR to validate the toxicity mechanism. After *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ treatment of zebrafish embryos for 96 hpf, 40 zebrafish larvae were selected at random from every treatment group and total RNA was extracted with Trizol according to the manufacturer's instructions (TransGen Biotechnology, Beijing, China). Reverse transcription of mRNA was performed to synthesise cDNA using a reverse transcription kit (TransGen Biotechnology, Beijing, China). A quantitative kit was then used to perform qRT-PCR (TransGen Biotechnology, Beijing, China), and *β*-actin was used as an internal control. Primer sequences were synthesised by Tsingke Biotech Co., Ltd. (Beijing, China), and their sequence details are shown in Supplementary Information Table S1.

2.10. Statistical analyses

The data are presented as mean ± standard error of the three independent replicates of each experiment. All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics 20.0 (IBM, USA). One-way analysis of variance, followed by post hoc Tukey's test, was utilised to identify substantial disparities among the respective groups. The significant difference symbols for group statistics were set as follows: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3. Results

3.1. Developmental responses to exposure to MEZ and its cis-isomers

Zebrafish embryos underwent exposure to various drug doses (0.02, 0.2, 2, and 4 mg L^{-1}) to facilitate a comparison of the developmental reactions to the exposure to *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ. The MEZ racemate and enantiomers appeared to cause delayed hatching and phenotypic defects in zebrafish embryos. No noticeable effects on the survival of zebrafish embryos after 96 hpf with *1S,5R*-MEZ were observed across the tested concentration range (Fig. 1A). The hatching rates measured at 48, 72, and 96 hpf after treatment with 4 mg L^{-1} of *1S,5R*-MEZ were 16.67%, 68.33%, and 88.25%, respectively (Fig. 1B). Further findings from the administration of *1S,5R*-MEZ revealed modifications in the overall body size and the eye area at 96 hpf. The eye area decreased to 0.05 mm^2 following treatment with 4 mg L^{-1} of *1S,5R*-MEZ (Fig. 1C). The length of the body in the groups treated with 4 mg L^{-1} was 3.22 mm, which was notably shorter than the 3.62 mm of the control groups (Fig. 1D). As illustrated in Fig. 1E, zebrafish embryos showed pericardial oedema after the course of treatment at 0.2, 2, and 4 mg L^{-1} of *1S,5R*-MEZ.

Zebrafish embryos were treated with 0.2, 2 and 4 mg L^{-1} *rac*-MEZ. Zebrafish embryos' survival rate was significantly lower when exposed to 4 mg L^{-1} of *rac*-MEZ than when exposed to *1S,5R*-MEZ at the same dose (Fig. 2A). Delayed hatching of embryos was also observed, where the hatching rate measured at 48, 72, and 96 hpf after 4-mg·L^{-1} *rac*-MEZ treatment was 3.42%, 66.67%, and 85.19%, respectively (Fig. 2B). At the highest concentrations of *rac*-MEZ (i.e., 4 mg L^{-1}), the eye area was reduced to 0.04 mm^2 and the body length decreased to 3.05 mm (Fig. 2C and D). Fig. 2E shows that the 0.2-, 2-, and 4-mg·L^{-1} treatment groups resulted in significant pericardial oedema in zebrafish embryos.

1R,5S-MEZ, another enantiomer of MEZ, exhibited a weaker survival rate following administration compared to *1S,5R*-MEZ and *rac*-MEZ group, particularly at 96 hpf (93.06% at 4 mg L^{-1}, Fig. 3A). Notably, a decline in the hatching rate was detected following treatment with 4 mg L^{-1} of *1R,5S*-MEZ at 48, 72, and 96 hpf. Additionally, a correlation between delayed hatching of embryos and decreased survival rates was observed. The phenomenon of developmental delay, whereby the eye area to 0.03 mm^2 and the body length was reduced to 2.82 mm, was

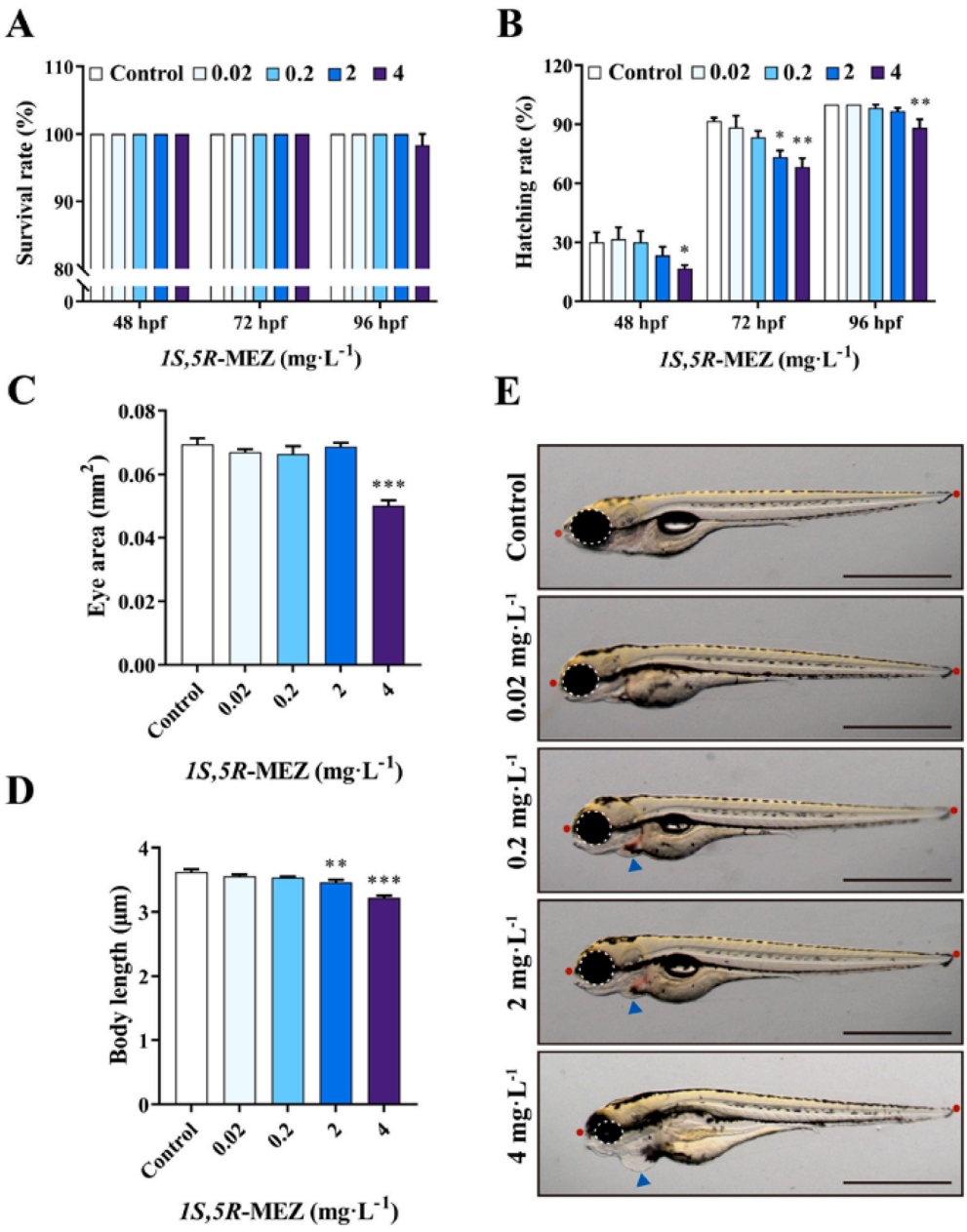

Fig. 1. Developmental toxicity of *1S,5R*-MEZ exposure to zebrafish larvae. The exposure concentrations were 0.02, 0.2, 2, and 4 mg L^{-1}. (A) Survival rate, n = 3, (B) hatching rate, n = 3, (C) eye area, n = 15, (D) body length, n = 15, and (E) representative image of *1S,5R*-MEZ-exposed larvae, the scale was 1000 μm *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control group.

observed after administering high concentrations of treatment, identical to that with *1S,5R*-MEZ and *rac*-MEZ treatments (Fig. 3C and D). The toxicity of *1R,5S*-MEZ was higher in relation to body length, eye area, and pericardial oedema (Fig. 3E).

Exposure to MEZ and its *cis*-isomers resulted in considerable developmental toxicity in zebrafish, with *1R,5S*-MEZ demonstrating the highest toxicity. Further investigations must be undertaken to establish the reasons for the variances in toxicity.

3.2. MEZ and its cis-isomers cause cardiac morphological and functional damage

After demonstrating MEZ's significant effect on pericardial oedema in zebrafish larvae, the *Tg(myl7:GFP)* line was employed to additionally validate the modifications in cardiac morphology and function that MEZ causes. According to our findings, the zebrafish larvae displayed pericardial oedema and linear heart stretching following exposure to *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ (0.02, 0.2, 2, and 4 mg L^{-1}) (Fig. 4A). The group exposed to 4-mg·L^{-1} MEZ exhibited the largest pericardial area of zebrafish larvae, with a rise in SV-BA distance observed in a dose-dependent manner (Fig. 4B and C).

We also assessed heart rate to evaluate the impact of MEZ on the cardiac function in zebrafish, considering that heart developmental problems impair blood circulation and lead to vascular malformations. In keeping with the changed cardiac morphology phenotype, heart rate was significantly decreased by the MEZ treatment (Fig. 4D). Examination of zebrafish hearts affected by pathology through H&E staining indicated that using 4 mg L^{-1} *rac*-MEZ and *1R,5S*-MEZ as treatment led to a decrease of cardiomyocytes and ventricular wall thinning (Fig. 4E).

Based on the cardiac phenotypic defects and functional abnormalities described above, *1R,5S*-MEZ demonstrated greater cardiotoxicity in zebrafish embryos compared to *1S,5R*-MEZ and *rac*-MEZ. To investigate

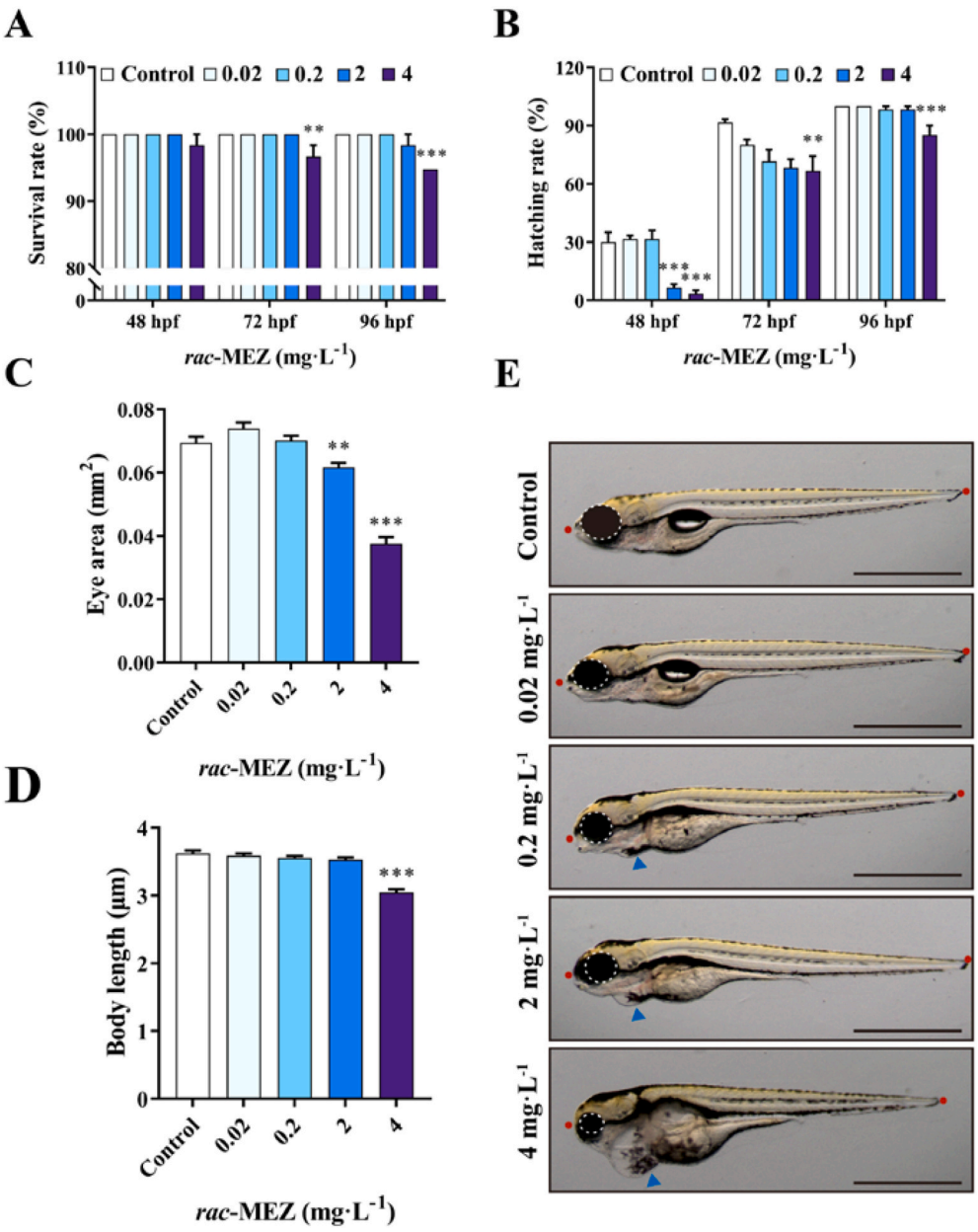

Fig. 2. Developmental toxicity of *rac*-MEZ exposure to zebrafish larvae. The exposure concentrations were 0.02, 0.2, 2, and 4 mg L^{-1}. (A) Survival rate, n = 3, (B) hatching rate, n = 3, (C) eye area, n = 15, (D) body length, n = 15, and (E) representative image of *rac*-MEZ-exposed larvae, the scale was 1000 μm *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control group.

the determinant factors underlying the observed variances in toxicity, six critical regulatory genes (*hand2*, *gata4*, *nkx2.5*, *tbx5*, *vmhc*, and *amhc*) were further evaluated. The *hand2*, *gata4*, *nkx2.5*, and *amhc* genes exhibited increases of 2.41-, 2.10-, 2.41-, and 2.48-folds, respectively, in zebrafish subjected to 4 mg-L^{-1} *1R,5S*-MEZ at 96 hpf, while the mRNA levels of *tbx5* and *vmhc* were reduced by 1.70- and 1.67-folds, compared to those in control-treated embryos (Fig. 4F). Additionally, the treatment of *rac*-MEZ resulted in increased expression of *hand2* and *gata4*, whereas treatment with *1S,5R*-MEZ did not produce any detectable modifications in gene expression that were statistically significant.

3.3. MEZ and its cis-isomers induce ISV numbers and vasotoxic effects

To investigate the vascular damage and developmental delay in zebrafish embryos caused by MEZ and its *cis*-isomers, we utilised the *Tg (kdrl:EGFP)* transgenic zebrafish. Each toxic substance (MEZ and its *cis*-isomers) was administered at concentrations of 4 mg L^{-1}, and the production of ISV in zebrafish was seen quantitatively using this transgenic zebrafish. Fig. 5A displays representative photos of individual embryos treated with 4 mg L^{-1} of *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ after 96 hpf. The number of ISV increased after exposure to *1R,5S*-MEZ ($p < 0.001$, Fig. 5B) and *rac*-MEZ ($p = 0.001$, Fig. 5B), while no such effects were observed with *1S,5R*-MEZ treatment.

Vascular development-associated gene expression was evaluated, and the results showed that the genes *dll4*, *vegfaa*, and *vegfc* showed increases of 5.58-, 3.84-, and 4.17-folds, in zebrafish exposed to *1R,5S*-MEZ, and the mRNA levels of *dll4*, *vegfaa*, and *vegfc* were increased of 3.00-, 2.01-, and 3.55-folds exposed to *rac*-MEZ, respectively, compared to control treated embryos, whereas *1S,5R-MEZ* had no observable effects on *vegfaa* and *vegfc* genes (Fig. 5C).

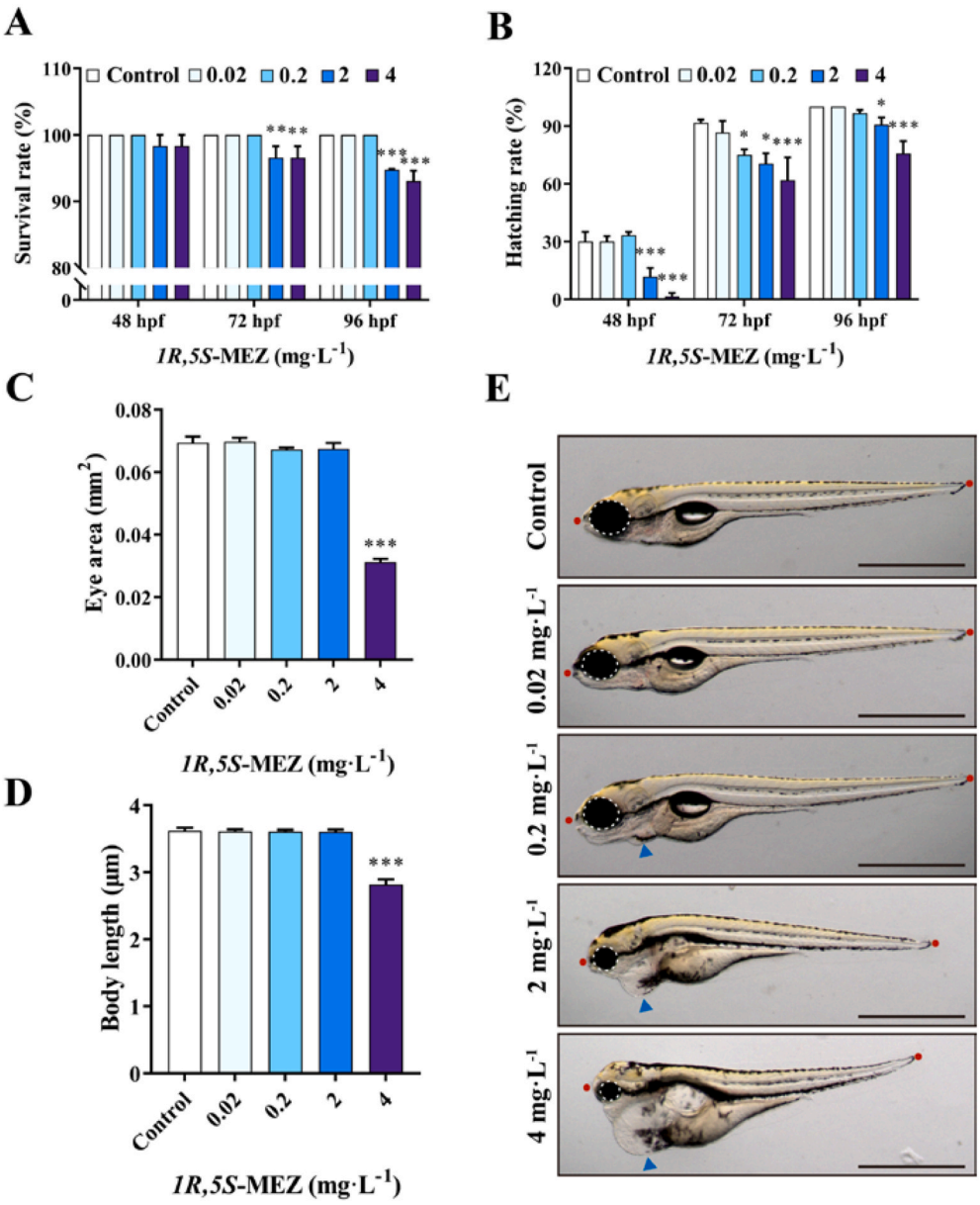

Fig. 3. Developmental toxicity of *1R,5S*-MEZ exposure to zebrafish larvae. The exposure concentrations were 0.02, 0.2, 2, and 4 mg L^{-1}. (A) Survival rate, n = 3, (B) hatching rate, n = 3, (C) eye area, n = 15, (D) body length, n = 15, and (E) representative image of *1R,5S*-MEZ-exposed larvae, the scale was 1000 μm *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control group.

3.4. MEZ and its cis-isomers exposure induced oxidative stress and apoptosis

Oxidative stress is commonly linked to cardiovascular toxicity. To clarify the possible mechanisms responsible for developmental anomalies caused by MEZ and its enantiomers, we analysed the accumulation of ROS and enzyme activity after 96-h treatment (Fig. 6). To assess ROS formation in more detail, DCFH-DA staining was employed (Fig. 6A). The *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ (4 mg L^{-1}) treatment groups showed a significant increase in ROS accumulation, with increases of 3.88-, 4.40-, and 5.06-fold, respectively (Fig. 6B). Furthermore, *rac*-MEZ, and *1R,5S*-MEZ exhibited significantly higher levels of SOD activity, malondialdehyde (MDA) and nitrous oxide (NO) concentration than did the control group (Fig. 6C–S2, and S3). The gene expression levels of antioxidantenzymes (*sod1* and *cat*) were examined. The gene of *sod1* increased by 1.55-, 1.66-, and 1.97-fold in the *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ treatments, respectively (Fig. 6D). Meanwhile, the expression levels of *cat* in the *1R,5S*-MEZ treatment increased by 2.60-fold (Fig. 6E). In contrast, treatment with *1S,5R*-MEZ and *rac*-MEZ yielded no statistically significant alterations in *cat* expression (Fig. 6E).

ROS accumulation is known to induce apoptosis; therefore, apoptotic cells were detected using AO staining (Fig. 6F). The *rac*-MEZ and *1R,5S*-MEZ treatment groups showed the highest levels of apoptosis, which were primarily localised in the heart region. The bax proteins were also assessed, which rose 2.98- and 3.82-fold after treatment with 4-mg·L^{-1} *rac*-MEZ and *1R,5S*-MEZ, respectively (Fig. 6G).

In summary, our findings indicated that *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ induced oxidative stress in zebrafish. However, the *rac*-MEZ and *1R,5S*-MEZ treatments showed more pronounced oxidative stress-induced apoptosis. Thus, oxidative stress and apoptosis may be responsible for variations in cardiovascular toxicity caused by MEZ and its *cis*-isomers.

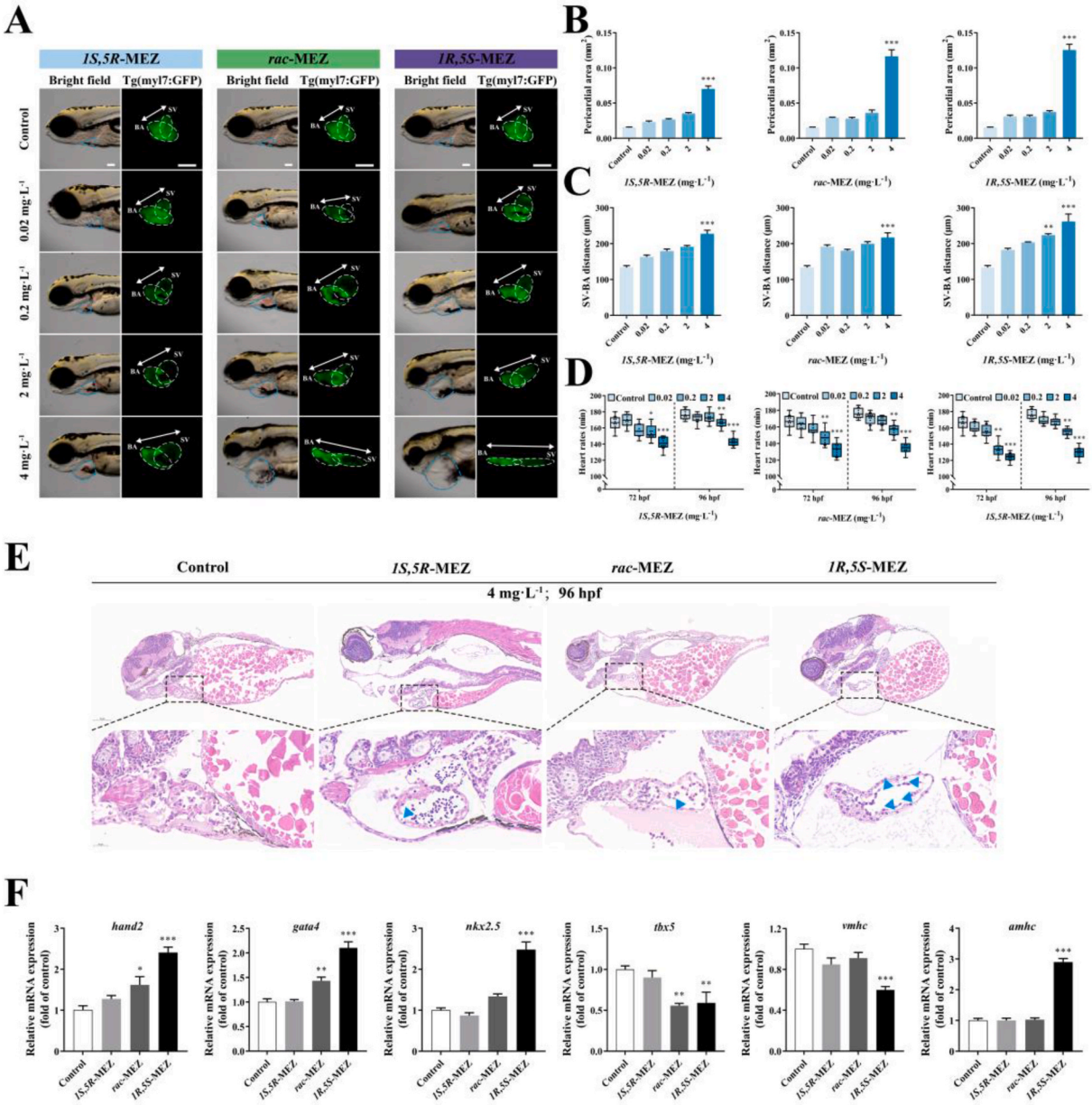

Fig. 4. Cardiac defects of zebrafish (*Tg(myl7:GFP)*) induced by *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ. The exposure concentrations were 0.02, 0.2, 2, and 4 mg L^{-1}, respectively. (A) Typical images of heart in transgenic zebrafish *Tg(myl7:GFP)*, scale bars correspond to 100 μm, (B) pericardial area, n = 15, (C) SV-BA distance heart rate, n = 15, (D) heart rate, n = 12, (E) H&E pathological sections, and (F) expression of cardiac development genes, such as *hand2*, *gata4*, *nkx2.5*, *tbx5*, *vmhc* and *amhc*. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control group.

3.5. ISQ attenuates MEZ-induced cardiotoxicity by preventing oxidative stress

To examine the potential involvement of oxidative stress in MEZ-triggered cardiotoxicity, we evaluated whether ISQ, a natural antioxidant capable of reducing oxidative stress, could alleviate MEZ-induced cardiotoxicity. In comparison to the MEZ-only group, ISQ significantly inhibited ROS production induced by MEZ (Fig. 7A and B), reduced SOD activity, MDA and NO concentration (Fig. 7C–S2, and S3) and decreased *sod1* and *cat* genes expression (Fig. 7D and E). Interestingly, ISQ alleviated the cardiac toxicity caused by MEZ (Fig. 7F). After ISQ supplementation, body length, eye area, heart rate, pericardial area and BA-SV distance all approached control dimensions (Fig. 7G). Taken together, the results indicate that ISQ has potential as a therapeutic agent in mitigating MEZ-induced cardiotoxicity through its antioxidant properties.

3.6. Wnt/β-catenin signaling pathway in cardiac toxicity

The Wnt/β-catenin signaling plays a crucial role in the development of numerous cardiovascular pathologies. To investigate how MEZ affects Wnt/β-catenin signaling, we first analysed the levels of β-catenin, a pivotal protein within this pathway. After being treated with 4-mg·L^{-1} *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ, respectively, we observed a rise in β-catenin levels of 1.28-, 1.74-, and 2.46-fold (Fig. 8B). We examined the expression of other important genes to provide more proof that MEZ interferes with the Wnt signaling pathway. We discovered that *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ increased the levels of *β-catenin* (2.17-, 2.41-, and 2.58-folds), *wnt3* (2.37-, 2.67-, and 5.72-folds), and *gsk3β* (2.99-, 6.57-, and 7.93-folds) (Fig. 8C, D, and E). However, *anix2* gene expression was increased by 1.44-fold only in the *1R, 5S*-MEZ group (Fig. 8F). Consequently, we hypothesised that the Wnt pathway acted as a mediating factor for the cardiac dysplasia caused by MEZ in zebrafish.

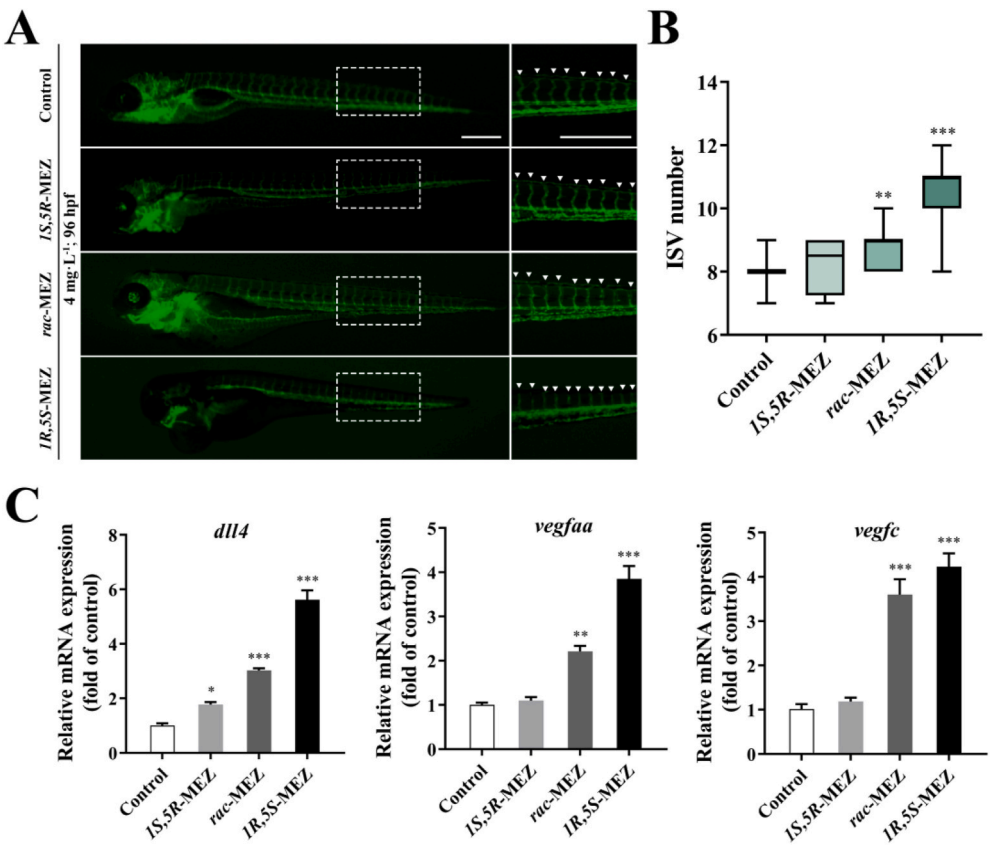

Fig. 5. Malformation of zebrafish (*Tg(kdrl:EGFP)*) vasculature after 96 hpf of induction by 4 mg L^{-1} *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ. (A) ISV number counts and images of vasculature, scale bars correspond to 500 μm, (B) ISV number of *Tg (kdrl:EGFP)* embryos, n = 20, and (C) *1S,5R*-MEZ-, *rac*-MEZ-, and *1R,5S*-MEZ-caused alterations in the expression levels of angiogenesis-associated genes, such as *dll4*, *vegfaa*, and *vegfc*. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared with the control group.

We used IWR-1, an inhibitor of the Wnt signaling pathway, to treat zebrafish to confirm this. We discovered that IWR-1 dramatically decreased MEZ-induced cardiotoxicity at 96 hpf (Fig. 8A). Additionally, the study analysed the genes linked with the Wnt signaling pathway following IWR-1 application. The results indicate that IWR-1 effectively ameliorated the abnormal gene levels associated with the Wnt signaling pathway induced by MEZ (Fig. 8C, D, E, and F).

4. Discussion

Although previous research has indicated the ability of *cis*-MEZ to cause stereoselective acute toxicity in zebrafish (He et al., 2022), gaps remain in our understanding of its cardiovascular toxicity and the underlying mechanisms. Herein, our study aimed to assess the cardiovascular toxicity of MEZ and its *cis*-isomer in zebrafish. Meanwhile, we conducted a detailed examination of the molecular variances in cardiotoxicity between MEZ and its *cis*-isomer, emphasising oxidative stress, cardiovascular developmental gene expression and the Wnt/*β*-catenin signaling mechanism.

We discovered that treatment by *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ resulted in zebrafish cardiotoxicity, which was characterised by a slow heart rate, increased SV-BA distance, and increased pericardial area using transgenic zebrafish *Tg(myl7:GFP)*. Similar to the properties of MEZ, several other pesticides exert cardiotoxicity in zebrafish. For instance, difenoconazole induces cardiac malformations, including yolk sac oedema and pericardial oedema, and impacts zebrafish heart rates (Wang et al., 2023). In addition, prothioconazole, also a fungicide, causes a reduction in heart rate, induces cardiac malformations, and hinders cardiac cycle development in zebrafish (Sun et al., 2020). Meanwhile, *1R,5S*-MEZ and *rac*-MEZ can cause more severe cardiac phenotypic defects compared with *1S,5R*-MEZ. Six important genes associated with heart development-*hand2*, *gata4*, *nkx2.5*, *tbx5*, *vmhc*, and *amhc*-were further confirmed at the expression level. Through its negative regulation of fibronectin, the *hand2* signaling pathway is essential for controlling heart morphogenesis (Garavitoaguilar et al., 2010). Ventricular formation requires *nkx2.5* and *hand2* coordinated action (George et al., 2015), but their increased expression triggers alterations in heart architecture and atrioventricular differentiation. The *Tg(myl7:GFP)* showed notable morphological alterations in the heart and cardiomyocyte through H&E staining, which were most likely caused by the considerable upregulation of *nkx2.5* and *hand2* expression in the *1R, 5S*-MEZ and *rac*-MEZ-treated groups. *gata4* is a fundamental gene in cardiac differentiation and cardioblast migration, regulating several developmental processes, including the formation of atrial, ventricular, and atrioventricular valves (Jia et al., 2019; Zeisberg et al., 2005). Overexpression of *gata4* results in the disruption of *nkx2.5*, leading to anomalous cardiac morphology (Wan et al., 2021). Additionally, the *amhc* gene is crucial for the development of ventricular and atrial myocytes (Liu et al., 2019). The *vmhc* gene expresses the ventricular myosin heavy chain protein, which is restricted to the ventricle. *vmhc* serves as a vital marker for ventricular myocardium in endo-cardiogenesis. Previous studies have noted that abnormal *vmhc* expression notably impacts cardiac morphogenesis (Han et al., 2015), while *tbx5* is recognised as an important gene in vertebrate cardiogenesis (Lu et al., 2022). The expression of *tbx5* and *vmhc* genes was downregulated by *1R,5S*-MEZ exposure, which could be zebrafish's compensatory reaction to cardiotoxicity. In this study, MEZ exposure led to gene changes linked to cardiac development, suggesting possible cardiac insufficiency and abnormal cardiac differentiation. The *1S, 5R*-MEZ treatment group did not cause any statistically significant

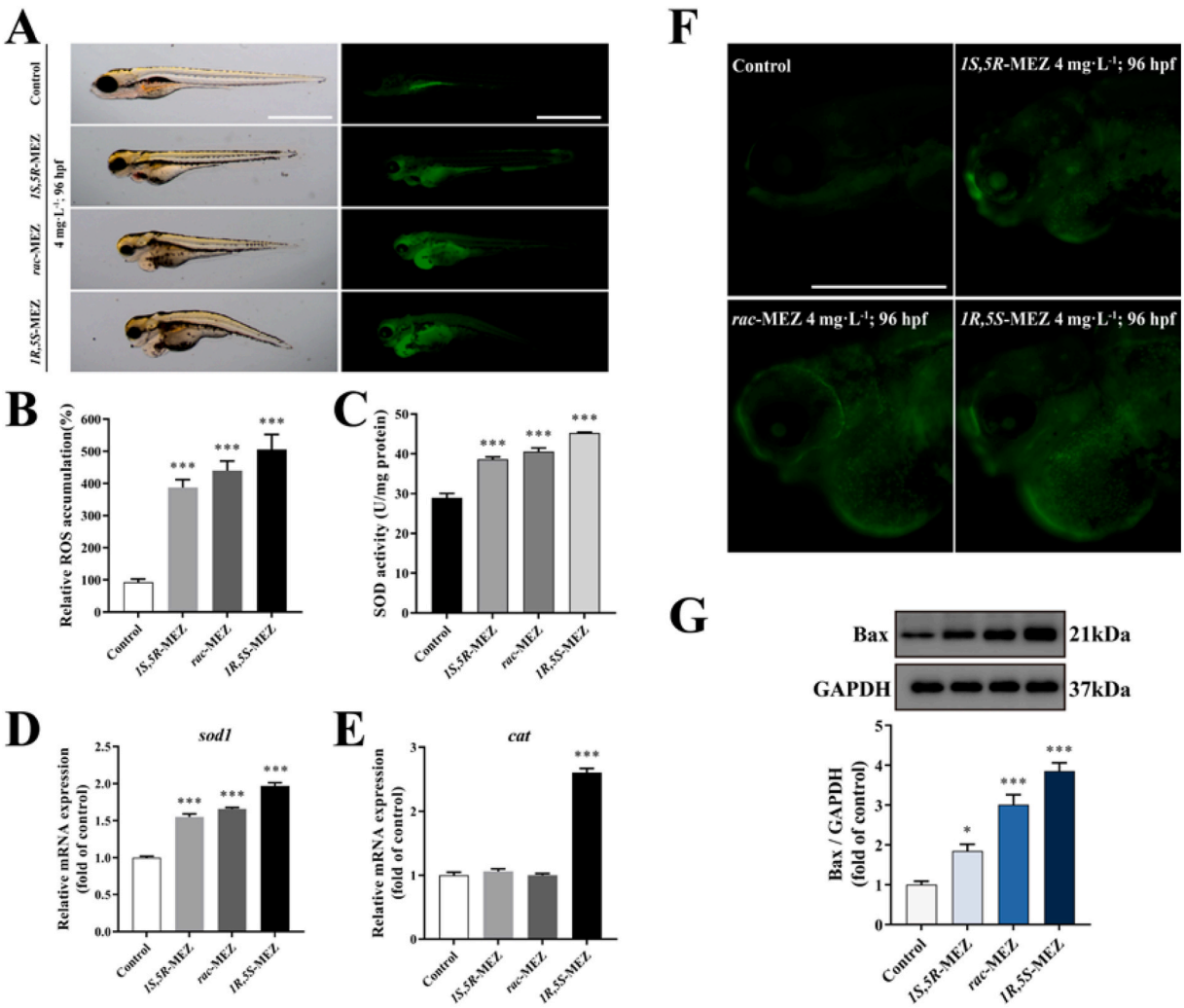

Fig. 6. *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ enhanced oxidative stress and apoptosis in zebrafish larvae. (A) The ROS accumulation after 4 mg L^{-1} *1S,5R*-MEZ, *rac*-MEZ and *1R,5S*-MEZ exposure was indicated by green fluorescence, scale bars correspond to 1000 μm, (B) the intensity of green fluorescence was analysed to identify the level of ROS accumulation, n = 20. (C) SOD activity, (D–E) relative mRNA expression of *sod1* and *cat*, (F) acridine orange (AO) staining, and (G) the bax protein was determined using Western blot analysis. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

changes in gene levels and displayed a lower impact on the heart, compared to the *1R,5S*-MEZ and *rac*-MEZ groups. These results suggest that exposure to MEZ during the initial phases of embryonic development results in abnormal gene regulation concerning cardiac function, eventually leading to the development of malformations in the heart. *1S, 5R*-MEZ has significant potential for commercial use as a *cis*-isomer of MEZ with high antimicrobial activity and low toxicity is being promoted.

To assess how MEZ affects vascular injury, we used *Tg(kdrl:EGFP)* zebrafish. Our discovery that *1R,5S*-MEZ and *rac*-MEZ have a significant impact on the total number of ISV is congruent with the effects of other types of fungicides. For instance, the number of ISV amplified when subjected to pyraclostrobin, another fungicide (Kim et al., 2021). Similarly, the number of ISV escalated as the treatment concentration increased when treated with 3-pyridinecarboxaldehyde (Cho et al., 2023). However, our study did not observe such effects with *1S,5R*-MEZ treatment. We also examined expression of genes directly associated with vascular development, focusing on *dll4*, *vegfaa*, and *vegfc*. The upregulation of these genes in the *1R,5S*-MEZ, and *rac*-MEZ-treated groups resulted in vascular damage. This finding concurs with a previous study which reported an increase in the expression of *dll4* and *vegfc* genes following exposure to the herbicide acetochlor and its chiral isomers, leading to vascular malformations (Wang et al., 2023). As vascular endothelial growth factor (VEGF) signals mediate vasculogenesis and angiogenesis (Liang et al., 2001), genes such as *vegfaa* have an integral role in angiogenesis during early embryogenesis of zebrafish (Rossi et al., 2016). DA growth is affected by *vegfc*-directed signalling, with *dll4* being a significant contributor to this phenomenon (Kwon et al., 2013; Niessen et al., 2011). Thus, our results strongly suggest that MEZ disrupts the morphology and function of the vascular system by upregulating the genes linked with vascular development.

As environmental pollutants can induce cardiovascular toxicity through oxidative stress damage (Cao et al., 2018), our study investigated the level of oxidative stress in MEZ-treated zebrafish embryos. Important indicators of oxidative stress include ROS, SOD, MDA, NO and CAT levels. In biological systems, oxidative stress involves a dynamic balance between the production and elimination of SOD and other antioxidant enzymes (Valavanidis et al., 2006). MDA commonly utilised as a marker for assessing oxidative damage induced by ROS (Ge et al., 2015). NO plays a key role in cellular oxidative stress and antioxidant defense mechanisms by modulating redox reactions. Dysregulation or deficiency of NO can result in oxidative stress, which may have detrimental cardiovascular effects on the organism (Takata et al., 2020; Carlstrom and Montenegro, 2018). The increased expression of ROS,

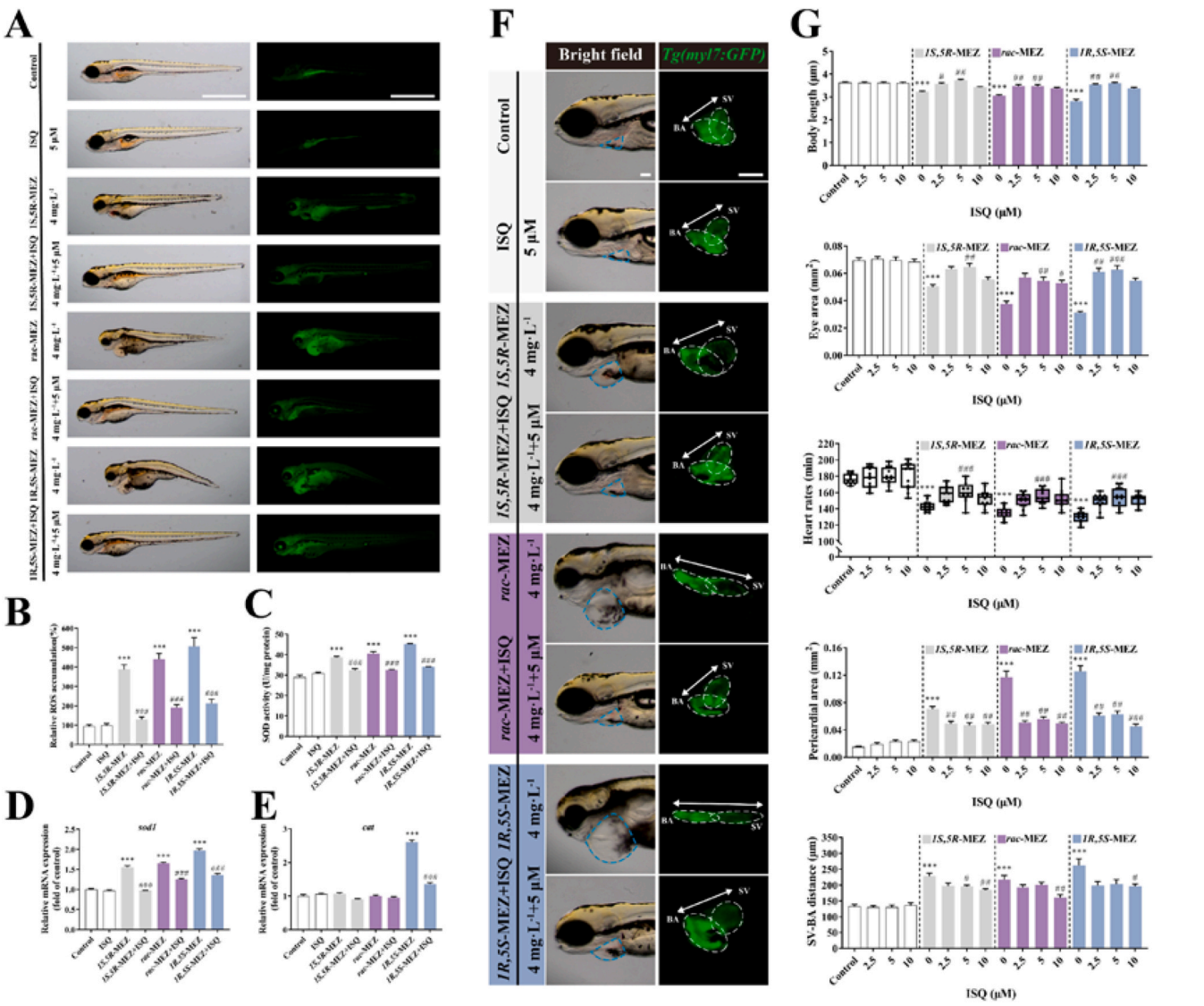

Fig. 7. ISQ attenuates oxidative stress in zebrafish after MEZ exposure. Zebrafish embryos were exposed to 4 mg L^{-1} *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ with or without 5 μM ISQ for 96 h, then cardiac development was evaluated in zebrafish larvae. (A) ROS accumulation was indicated by green fluorescence, scale bars correspond to 1000 μm, (B) the intensity of green fluorescence was analysed to identify the level of ROS accumulation, n = 20, (C) SOD activity, (D–E) relative mRNA expression of *sod1* and *cat*, (F) typical images of the heart in transgenic zebrafish *Tg(myl7:GFP)*, scale bars correspond to 100 μm, and (G) quantification of developmental and cardiac phenotype-related parameters, including body length, eye area, heart rate, pericardial area, and SV-BA distance, n = 15. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control group. #P < 0.05, ##P < 0.01, ###P < 0.001, compared with the *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ treatment group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

SOD, MDA, and NO in zebrafish larvae exposed to MEZ confirms the induction of oxidative stress, consistent with alterations in antioxidant genes like *sod1* and *cat*. These findings suggest a potential association between MEZ exposure and oxidative stress.

In addition, the accumulation of excess ROS damages mitochondrial activity, which in turn promotes apoptosis (Chowdhury et al., 2009). Therefore, using AO staining, we observed apoptosis brought on by oxidative stress in living zebrafish, triggered by *1S,5R*-MEZ, *rac*-MEZ, and most severely by *1R,5S*-MEZ. These findings indicate that oxidative stress-induced apoptosis may contribute to cardiovascular abnormalities in zebrafish.

To further confirm the contribution of oxidative stress to cardiotoxicity, we examined the cardiac development of the zebrafish group exposed to MEZ after suppressing oxidative stress with the ISQ antioxidant. The results indicated that ISQ reduced MEZ-induced oxidative stress, potentially repairing cardiotoxicity and developmental defects. This finding suggests that ROS likely caused impaired embryonic and cardiac development in the MEZ-treated zebrafish group.

In the Wnt/*β*-catenin signaling pathway, *β*-catenin undergoes rapid phosphorylation and degradation via CK1 as well as the adenomatous polyposis (APC)/axin/glycogen synthase kinase (GSK)-3*β*-complex (Bertozzi et al., 2022; Yue et al., 2017; Perugorria et al., 2019). The activation of the Wnt/*β*-catenin signaling pathway in zebrafish embryos during early development results in heart defects (Hurlstone et al., 2003). Our results showed a significant upregulation of the Wnt-pathway-related genes *β-catenin*, *wnt3*, *gsk3β*, and *axin2* following MEZ exposure. The *β*-catenin level, a crucial protein in the Wnt signaling pathway, was also elevated. The Wnt/*β*-catenin signaling pathway inhibitor, IWR-1, decreased gene expression related to the Wnt/*β*-catenin signaling pathway and successfully restored heart abnormalities in zebrafish. Thus, we demonstrated that MEZ activates the Wnt pathway, triggering cardiotoxicity in zebrafish. Similar to our findings, studies on benzophenone and adriamycin have linked cardiotoxicity to the upregulating of the Wnt signaling pathway in zebrafish embryos (Zuo et al.,

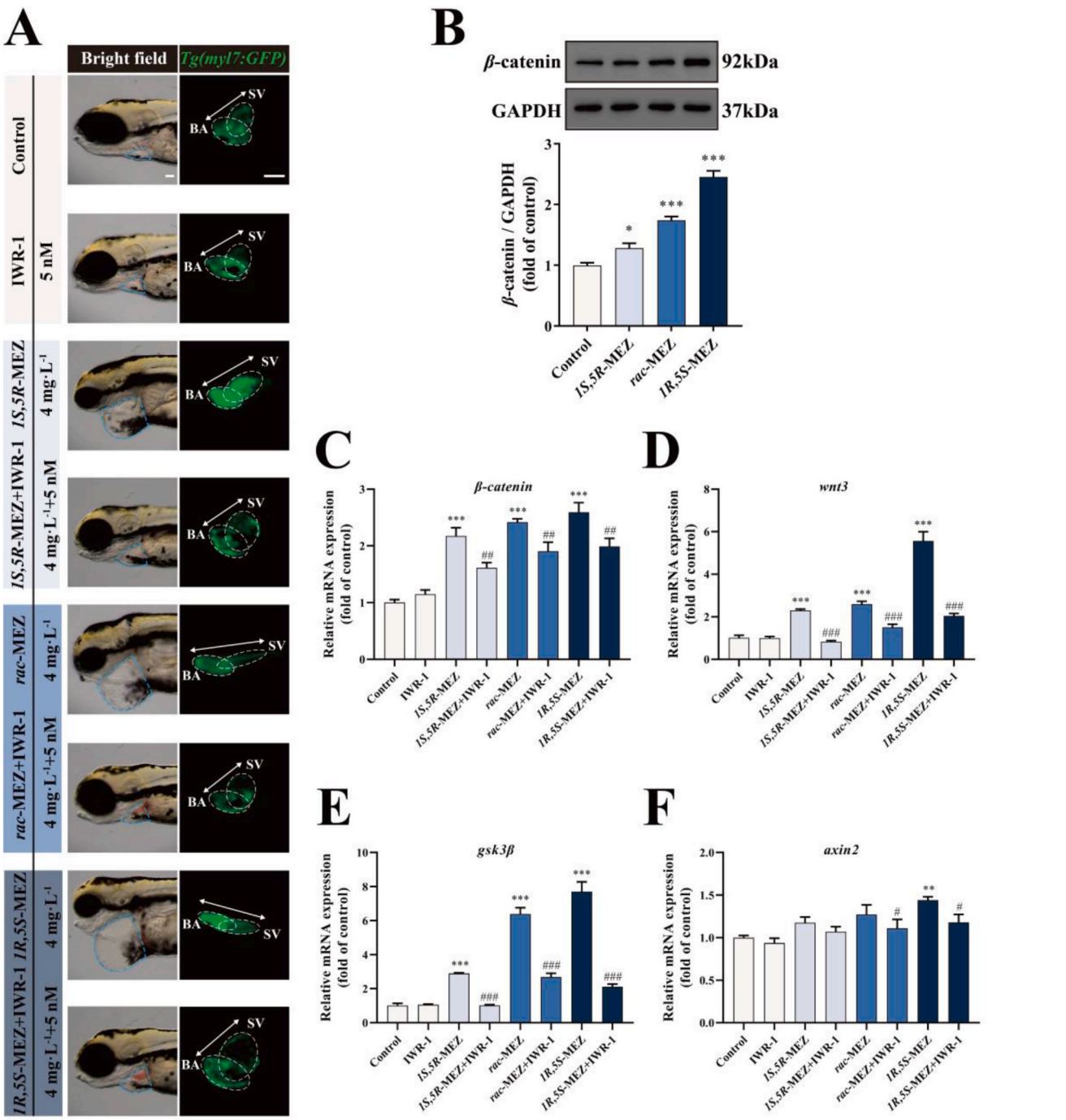

Fig. 8. IWR-1 attenuates cardiac toxicity in zebrafish after MEZ exposure. Zebrafish embryos were exposed to 4 mg L^{-1} *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ with or without 5 nM IWR-1 for 96 h. (A) Typical heart images in transgenic zebrafish *Tg(myl7:GFP)*, scale bars correspond to 100 μm, (B) Western blot analysis of *β*-catenin in zebrafish, and (C–F) mRNA levels of 4 genes involved in the Wnt signalling pathway analysed by qRT-PCR.*P < 0.05, **P < 0.01, and ***P < 0.001, compared with the control group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001, compared to the *1S,5R*-MEZ, *rac*-MEZ, and *1R,5S*-MEZ treatment group.

2023; Duan et al., 2023). However, some drug exposures resulted in varying effects. For example, triclosan downregulates Wnt signaling, yet cardiac defects induced by triclosan are reportedly rescued by activating Wnt signaling (Diao et al., 2023). Additionally, difenoconazole, a triazole fungicide, inhibits the Wnt/*β*-catenin pathway by activating peroxisome proliferative activity receptor *γ*, leading to an asymmetrical heart development (Wang et al., 2023). Although difenoconazole and MEZ are triazole fungicides, their effects on the Wnt/*β*-catenin pathway vary significantly. Agrochemicals' interference with the Wnt/*β*-catenin pathway may cause this discrepancy, as their production of cardiotoxicity is not confined to a single route, unlike knockdown/overexpression (Yang et al., 2023). Consequently, these differences may have an unanticipated impact on the tightly controlled process of heart development.

In summary, our comprehensive assessment compared MEZ and its *cis*-isomers, examining their impact on developmental and cardiovascular aspects in zebrafish. Furthermore, our study demonstrated that MEZ-induced cardiac abnormalities could be mitigated by introducing natural antioxidants like ISQ and inhibitors of the Wnt signaling pathway. Hence, MEZ potentially induces cardiotoxicity in zebrafish

embryos due to cardiovascular insufficiency and anomalous expression of cardiovascular-related genes mediated by the Wnt/β-catenin signaling pathway and oxidative stress. The cardiotoxic effects of environmental pollutants on zebrafish are similar to those observed in other fish species, including tilapia (Ivantsova et al., 2024), rainbow trout alevins (Eriksson et al., 2022), and medaka (Liu et al., 2024). Therefore, it is plausible to hypothesised that MEZ and its *cis*-enantiomers could potentially induce cardiotoxicity in other fish species. Notably, ISQ has shown excellent ability to mitigate cardiotoxicity by inhibiting oxidative stress. However, the effects of ISQ on genes related to cardiovascular development are unknown. Thus, the mechanism of mitigating toxicity of ISQ warrant further study.

5. Conclusions

This study is among the limited number of investigations that examine the cardiovascular toxicity caused by MEZ and its *cis*-isomers, revealing the mechanisms responsible for such toxicity. When exposed to MEZ and its *cis*-isomers during the embryonic stage, the heart developed and functioned abnormally stereoselectively. This was also accompanied by increased production of ROS and cardiomyocyte apoptosis. Mechanically, MEZ and its *cis*-isomers upregulate the genes in the Wnt/β-catenin pathway and change the regulation of genes important in cardiovascular development. Further, the study identifies *1R,5S*-MEZ as the major contributor to embryo toxicity in zebrafish. This study establishes the foundation for future research in this field and provides a basic understanding regarding the toxicity mechanisms of MEZ and its *cis*-isomers during the development of the cardiovascular system. Furthermore, our research offers treatment options and targets for cardiovascular disorders brought on by environmental contaminants.

CRediT authorship contribution statement

Lulu Liu: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Fengzhong Wang:** Project administration, Funding acquisition. **Zhong Zhang:** Project administration, Funding acquisition. **Bei Fan:** Project administration, Funding acquisition. **Ying Luo:** Validation, Investigation. **Lin Li:** Validation, Investigation. **Yifan Zhang:** Validation, Investigation. **Zhihui Yan:** Validation, Investigation. **Zhiqiang Kong:** Validation, Methodology, Investigation, Conceptualization. **Frédéric Francis:** Validation, Methodology, Investigation, Conceptualization. **Minmin Li:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.124034.

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