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α-asarone induces cardiac defects and QT prolongation through mitochondrial apoptosis pathway in zebrafish

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Highlights

- α-asarone has adverse effects on the cardiac development in zebrafish embryos
- α-asarone prolongs the mean QTc duration and causes smaller amplitude of T-wave
- The expression of heart development-related genes are disrupted by α-asarone
- α-asarone triggers apoptosis in the zebrafsh heart
- α-asarone induces cardiotoxicity through mitochondrial apoptosis pathway

Abstract:

α-asarone is a natural phenylpropene found in several plants, which are widely used for flavoring foods and treating diseases. Previous studies have demonstrated that α-asarone has many pharmacological functions, while some reports indicated its toxicity. However, little is known about its cardiovascular effects. This study investigated developmental toxicity of α-asarone in zebrafish, especially the cardiotoxicity. Zebrafish embryos were exposed to different concentrations of α-asarone (1, 3, 5, 10, and 30 μM). Developmental toxicity assessments revealed that α-asarone did not markedly affect mortality and hatching rate. In contrast, there was a concentration-dependent increase in malformation rate of zebrafish treated with α-asarone. The most representative cardiac defects were increased heart malformation rate, pericardial edema areas, sinus venosus-bulbus arteriosus distance, and decreased heart rate. Notably, we found that α-asarone impaired the cardiac function of zebrafish by prolonging the mean QTc duration and causing T-wave abnormalities. The expressions of cardiac development-related key transcriptional regulators *tbx5*, *nkx2.5*, *hand2,* and *gata5* were all changed under α-asarone

exposure. Further investigation addressing the mechanism indicated that α-asarone triggered apoptosis mainly in the heart region of zebrafish. Moreover, the elevated expression of *puma*, *cyto C, afap1, caspase 3,* and *caspase 9* in treated zebrafish suggested that mitochondrial apoptosis is likely to be the main reason for α-asarone induced cardiotoxicity. These findings revealed the cardiac developmental toxicity of α-asarone, expanding our knowledge about the toxic effect of α-asarone on living organisms.

Keywords: cardiotoxicity; QT duration; T-wave; apoptosis; zebrafish

1. Introduction

α-asarone is a natural phenylpropenes, isolated from a number of plants, such as *Acorus calamus* and *Acorus gramineus* [1]. *Acorus calamus*, commonly known as sweet flag, has been widely used in traditional Indian and Chinese medicine [2]. It is also used for flavoring food, dietary supplements, and in phytopharmaceuticals [3, 4]. It has been reported that α-asarone possesses a wide range of beneficial pharmacological properties, which can be potentially used to treat various diseases [5] including epilepsy, hyperlipidemia, and respiratory disorders [6-8]. However, β-asarone, an isomer of α-asarone with the same formula of $C_{12}H_{16}O_3$, is not recommended for the clinical use because of its toxicity [5]. β-asarone has a cis structure, while α-asarone has a trans structure, which is generally more stable than the cis-structure. In animals, β-asarone can be metabolized to α-asarone [9].

Despite of the wide-spread use of α-asarone, only few studies investigated its toxicity. For example α-asarone exhibited genotoxicity and hepatocarcinogenicity in rodents [10, 11]. The bone marrow samples of BaLB/C mice treated with α-asarone displayed an increased sister chromatid exchange and reduced mitotic index [12]. α-asarone treatment caused DNA damage in human lymphocytes [12], rat hepatocytes [13], L929 cells [10], and hamster lung fibroblasts V79 cells [14]. These findings ~~verified~~ confirmed the genotoxic effect of α-asarone. Besides, the morphology of adult rat hepatocytes was altered under α-asarone administration [15]. It also had toxic effect on human hepatocytes THLE-2 cells [16]. In addition, reproductive toxicity induced by α-asarone has been reported. The sperm concentration, sperm motility, and seminal vesicle weight were reduced by α-asarone in CF1 mice [17]. α-asarone treatment in mice resulted in fetal malformations including hydrocephaly, extra-ribs, club feet and cleft lips [18]. However, little is known about the developmental toxicity of α-asarone, especially its cardiotoxicity.

Zebrafish is deemed as a great vertebrate model for toxicity assessment [19-24]. The cardiovascular, nervous, and visual systems of zebrafish are similar to that of mammalian at the physiological and molecular levels [25-28]. The concordance (positive and negative) between zebrafish embryos and mammalian models of developmental toxicity was reported to be ranging from 64% to 100% [29, 30]. Therefore, we can predict chemicals potential toxicity in humans through zebrafish toxicity assessment. In addition, the development of zebrafish embryos is not completely dependent on the functional cardiovascular system. They can survive the first 7 days without cardiovascular function, allowing assessing severe cardiovascular defects in zebrafish.

Apoptosis is a process of programmed cell death, which maintains cellular homeostasis between cell division and cell death [31]. It has been proved that α-asarone plays a vital role in promoting human Eca-109 cell apoptosis [32]. Moreover, previous study reported that β-asarone, another bioactive phytochemical in the *Acorus calamus*, can induce apoptosis through the mitochondrial apoptosis pathway by decreasing the Bcl-2/Bax ratio and activating Caspase 9 and Caspase 3 cascades in colon cancer cells [33]. Therefore, we speculated that α-asarone probably triggers apoptosis in cardiomyocytes ~~producing~~ resulting in heart malfunction.

In the present study, we first examined the developmental defects of the embryo caused by α-asarone, including mortality, hatching rate, and malformation rate. Then, the indicators of cardiotoxicity included the heart malformation, pericardial edema, SV-BA distance, heart rate, and QT interval were detected. Additionally, the expressions of cardiac development-related key transcriptional regulators were tested. The apoptosis in the heart was studied emphatically from morphological and molecular aspects. We further investigated the possible mechanism underlying α-asarone-induced apoptosis, particularly focusing on the mitochondrial apoptosis pathway.

2. Materials and Methods

2.1 Chemicals and reagents

We purchased α-asarone, methylene blue, tricaine, 1-phenyl-2-thiourea (PTU), potassium acetate, and acridine orange from Sigma (St. Louis, USA). The stock

solutions were prepared in double-distilled water or dimethyl sulfoxide (DMSO). We prepared the serial dilutions in bathing medium before the experiments.

2.2 Animal maintenance and drug exposure

Wild type zebrafish (AB) and cardiac myosin light chain 2 (cmlc2) transgenic zebrafish line *Tg* (*cmlc2:GFP*) were maintained according to standard procedures [34]. We collected embryos from natural mating of adult zebrafish bred and transferred embryos in bathing medium containing 2 mg/L methylene blue. At 4 hours post fertilization (hpf), zebrafish embryos were examined under a dissecting microscope (Olympus, Tokyo, Japan). We selected embryos which developed normally to the blastula stage for subsequent experiments. 0.003% PTU was added to the bathing medium after 10-12 hpf to inhibit melanin formation.

For α-asarone treatment, we randomly distributed the normal embryos (4 hpf) into six-well plates. Then embryos were treated with various concentrations of α-asarone (1, 3, 5, 10, and 30 μM). The different concentrations were selected based on preliminary findings. Specifically, the LC_1 and LC_{50} of α-asarone at 144 hrs are 3.4 μM and 32.7 μM, respectively (Figure 1B). To investigate the toxic and dose-response effect of α-asarone, we selected two concentrations below LC_1 (1 μM and 3 μM) and three concentrations between LC_1 and LC_{50} (5 μM, 10μM, and 30 μM). The untreated embryos were used as the control. Three replicates were run for each group and all tests were repeated three times. We changed the medium once every 24 hrs at which time any dead embryos were discarded. All zebrafish embryos were kept at 28 ± 0.5 °C with a light and dark cycle.

2.3 Developmental toxicity assessment

The developmental toxicity of α-asarone was indicated by mortality, hatching rate, and malformation rate [26]. The mortality, which was examined from 24 hpf to 192 hpf, was identified by coagulation of the larvae and missing heartbeat. We examined the hatching rate at 48 hpf and 72 hpf. At 168 hpf and 192 hpf, we recorded embryo morphology and analyzed the malformation rate.

2.4 Cardiotoxicity assessment

Zebrafish embryos at 96 hpf were anesthetized following α-asarone treatment. We randomly selected 8 individuals from each group for visual observation and image acquisition. The heart malformation was identified by pericardial edema, abnormal circulation, thrombosis and hemorrhage. The looping of the heart tube was quantified by measuring the distance between the sinus venosus (SV) and bulbus arteriosus (BA) [35]. We counted the heart rate by visual observation in 30 s intervals [36] and measured the SV-BA distance and the pericardial sac area using Image Pro Plus software (Media Cybernetics, Bethesda, USA) [37]. For electrocardiography (ECG), we anesthetized α-asarone treated zebrafish (3 dpf) and transferred it to the testing plates. We used a glass micropipette with diameter less than 5 μm as an electrode, which was filled with 3 M potassium acetate. The electrode was placed at the heart area to collect electrical signals. We set up the ECG recording device (ELC-03XS; NPI Electronic; Tamm, Germany) according to the manufacturer's protocol with record duration of 5 minutes and sampling interval at 3 ms. The results were analyzed using Lab Chart 7 (Lab Chart; ADInstruments, Australia). The mean intervals for QT and corrected QT interval (QTc) were calculated as previous described [38].

2.5 Real-time quantitative PCR

After α-asarone exposure for 96 hrs, we collected zebrafish for real-time quantitative PCR (qPCR). We extracted total RNA from zebrafish larvae (n≥30) using EASY spin Plus RNA Mini Kit (Aidlab Biotechnologies; Beijing, China). Extracted RNA was reverse transcribed into cDNA using the PrimeScript™ RT Master Mix (Takara; Tokyo, Japan). We performed the real-time qPCR using SYBR® Premix DimerEraser™ (Takara, Tokyo, Japan). Runs were carried out in triplicate using Light Cycler® 96 System (Light Cycler® Instrument; Roche; Switzerland) and normalized to housekeeping gene *rpl13a*. We analyzed data using LC 96 Application Software and calculated the relative gene expression according to the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table S1.

2.6 Behavioral testing

Following α-asarone treatment for 96 hrs, we placed larvae from each group in 48-well plates with 1 larva per well and 500 μl of bathing medium. After a 15 min acclimation period, the locomotion of each larva was recorded for 20 min using an automated computerized video-tracking system [39]. We used Zeblab software (Viewpoint, Lyon, France) to analyze the digital tracks. Eight larvae (n = 8) were run for each group.

2.7 Apoptotic assessment

For apoptotic assessment, we used both acridine orange (AO) staining and terminal deoxynucleotide transferase-mediated UTPnick end labeling (TUNEL) assay. For AO staining, zebrafish treated with α-asarone for 96 hrs were washed twice with PBS, and incubated with 5 μg/mL AO for 20 min in the dark at room temperature. Then the

larvae were washed with PBS for three times [40] and photographed by using a microscope (Zeiss, Jena, Germany). For TUNEL assay, zebrafish treated with α-asarone for 96 hrs were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight. After washing with PBS, endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in methanol for 15 min at room temperature. The larvae were washed thoroughly with PBS for twice and incubated with TUNEL reaction mixture (One Step TUNEL Apoptosis Assay Kit, Beyotime, Jiangsu, China). After incubation at 37 °C for 60 min, we washed the zebrafish for two times and then performed microscopy (Zeiss, Jena, Germany).

2.8 Immunohistochemistry

We carried out immunohistochemistry on heart cryostat sections (16 um) according to standard protocols [41]. In brief, zebrafish exposed to α-asarone for 96 hrs were fixed in 4% PFA at 4 °C overnight. Fixed zebrafish were rinsed in PBS and equilibrated in 30% sucrose before embedding in Tissue-Tek OCT compound (Sakura Finetek Europe B.V.). The primary antibody used in this study is rabbit anti-Caspase 3 at 1:200 (Proteintech) and the secondary antibodies used (1:200) was Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch). We performed microscopy using a Zeiss LSM 510 confocal microscope.

2.9 Statistical analysis

The results were analyzed by one-way ANOVA followed by Dunnett's post-hoc test using Graph Pad Prism 5.0 (GraphPad Software; CA, USA) and expressed as mean ± SEM. $P<0.05$ was considered as significant.

3. Results

3.1 Developmental toxicity of α-asarone in zebrafish

The zebrafish embryos treated with α-asarone from 24 hpf to 72 hpf (Figure 1A) exhibited normal development as the control. The hatching rate was markedly decreased in zebrafish exposed to 30 μM of α-asarone at ~~24~~ 48 hpf, while there was no significant difference in the percentage of hatching at 72 hpf among untreated and zebrafish treated with different concentrations of α-asarone (Figure 1C). To investigate the developmental toxicity of α-asarone, we tested its toxic effect from 24 hpf to 192 hpf since zebrafish can survive the first 168 hrs without cardiovascular function. An apparent increase in mortality was observed in 30 μM α-asarone treated group from 24 hpf to 192 hpf (Figure 2B). We also recorded the α-asarone induced phenotypic defects and the malformation rate of larval zebrafish at 168 hpf and 192 hpf (Figure 2A and 2C). The malformation rate was significantly elevated in the groups with the concentrations higher than 3 μM in a concentration dependent manner. The group treated with 30 μM of α-asarone had a 100% malformation rate. The phenotypic defects included the absence of a swimming bladder, yolk retention, curved body shape, and abnormal cardiac morphology. Moreover, only the group with 30 μM of α-asarone showed apparent difference in locomotor activity when compared with the control (Figure S1).

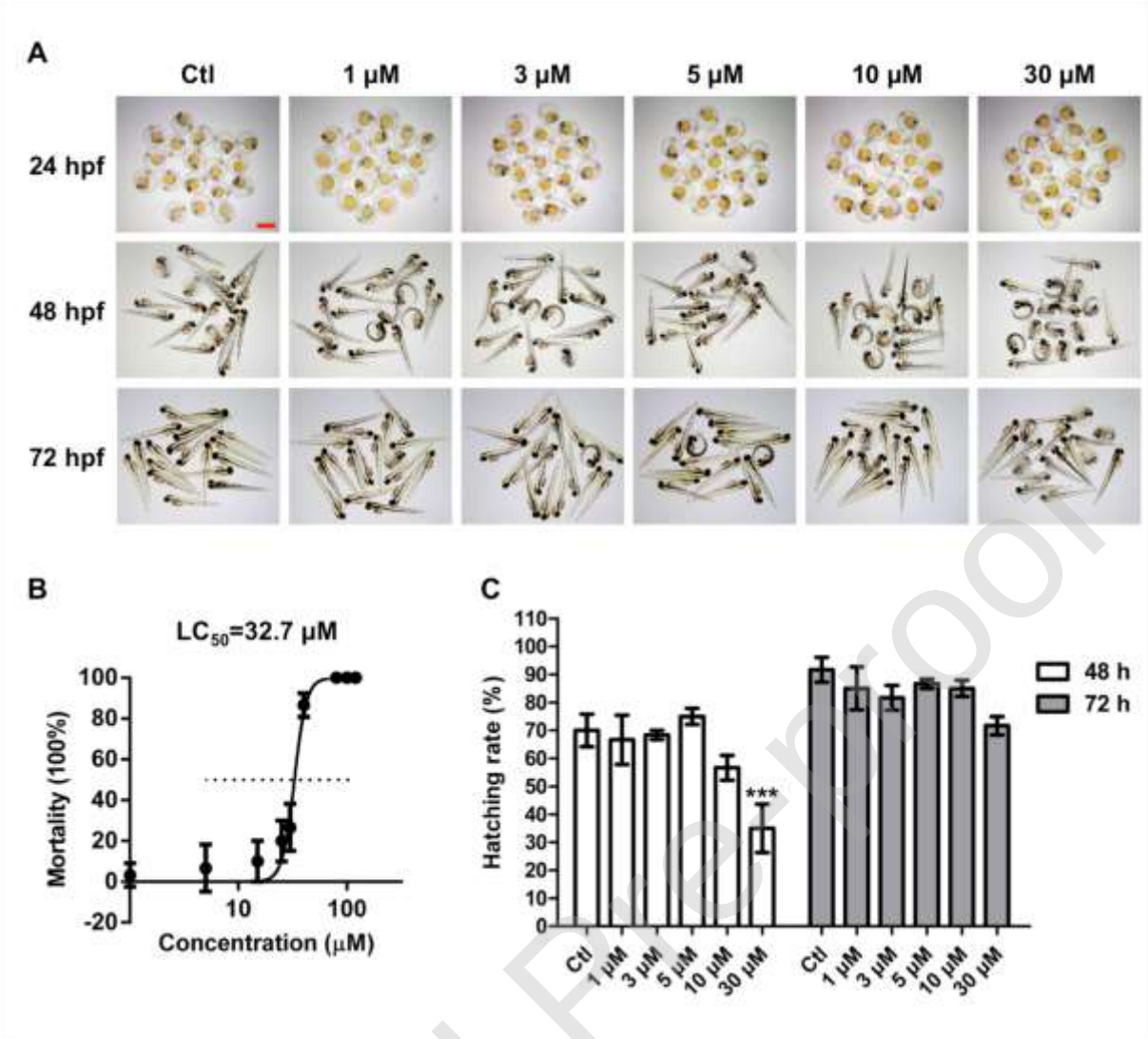

Figure 1. LC_{50} and hatching rate of zebrafish larvae treated with α-asarone. (A) Representative images of the morphology of zebrafish embryos exposed to 1, 3, 5, 10, and 30 μM α-asarone at 24，48 and ~~96~~ 72 hpf. (B) LC_{50} and (C) Hatching rate of the embryos treated with 1, 3, 5, 10, and 30 μM α-asarone. n=10 per group and the tests were repeated 3 times. Scale bar, 1000 μm. ***P < 0.001 vs Ctl.

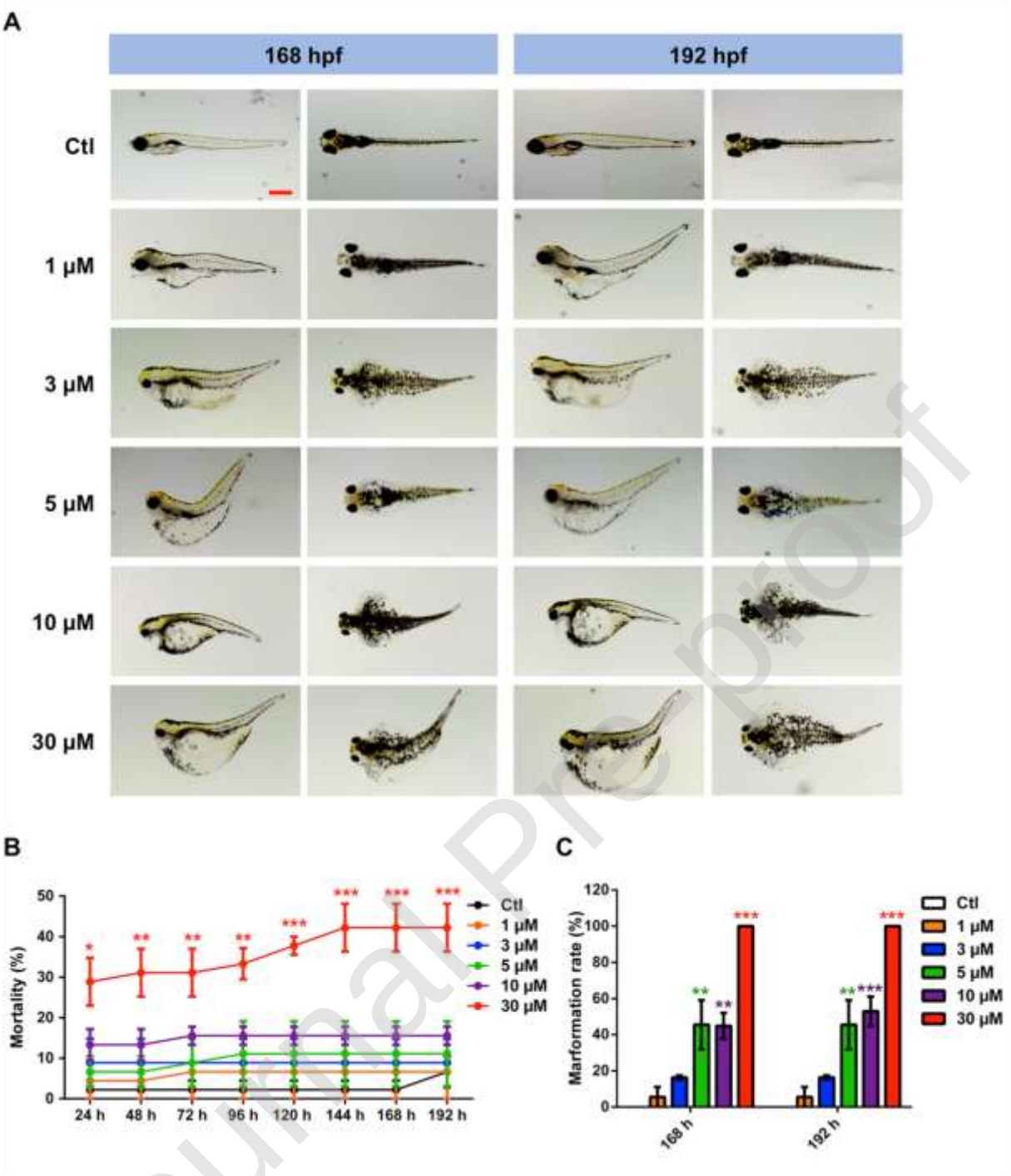

Figure 2. Developmental toxicity of zebrafish larvae after exposure to different concentrations of α-asarone. (A) The phenotypic defects of the zebrafish exposed to different concentrations of α-asarone at 168 hpf and 192 hpf. (B) Mortality rate of zebrafish treated with 1, 3, 5, 10, and 30 μM α-asarone from 24 hpf to 192 hpf. (C) Malformation rate of α-asarone treated zebrafish at 168 hpf and 192 hpf. Scale bar,

500 μm. *P < 0.05; **P < 0.01; ***P < 0.001 vs Ctl.

3.2 α-asarone caused cardiac developmental toxicity

The most representative malformation of α-asarone treated zebrafish revealed in this study was pericardial edema (Figure 3A), suggesting that α-asarone has cardiotoxic effect during development. Thus, we examined the parameters associated with cardiotoxicity in zebrafish treated with α-asarone for 96 hrs. As a result, a significant and concentration-dependent increase in the heart malformation was observed (Figure 3B). Moreover, α-asarone caused severe pericardial edema at the concentrations higher than 3 μM in a concentration-dependent manner (Figure 3C).

The SV-BA distance provides a marker for the development of the heart into two distinct chambers. Here, cardiomyocyte-specific zebrafish, which expressed EGFP driven by the *cmlc2* promoter [35] were used to evaluate the SV-BA distance [42]. We found that α-asarone strikingly increased the distance in all α-asarone treated groups as compared to the control (Figure 3D and 3E). The two chambers largely overlap with each other in a lateral view in the control, while the hearts were stretched out in the zebrafish exposed to α-asarone. Accordingly, after α-asarone treatment for 96 hrs, the larval zebrafish suffered from abnormal heart rhythm in a concentration-dependent manner (Figure 3F). No significant difference in the heart rate was found in the zebrafish exposed to 1, 3, and 5 μM of ~~α-Bisabolol~~ α-asarone, whereas 10 and 30 μM of α-asarone caused markedly decreased heart rate.

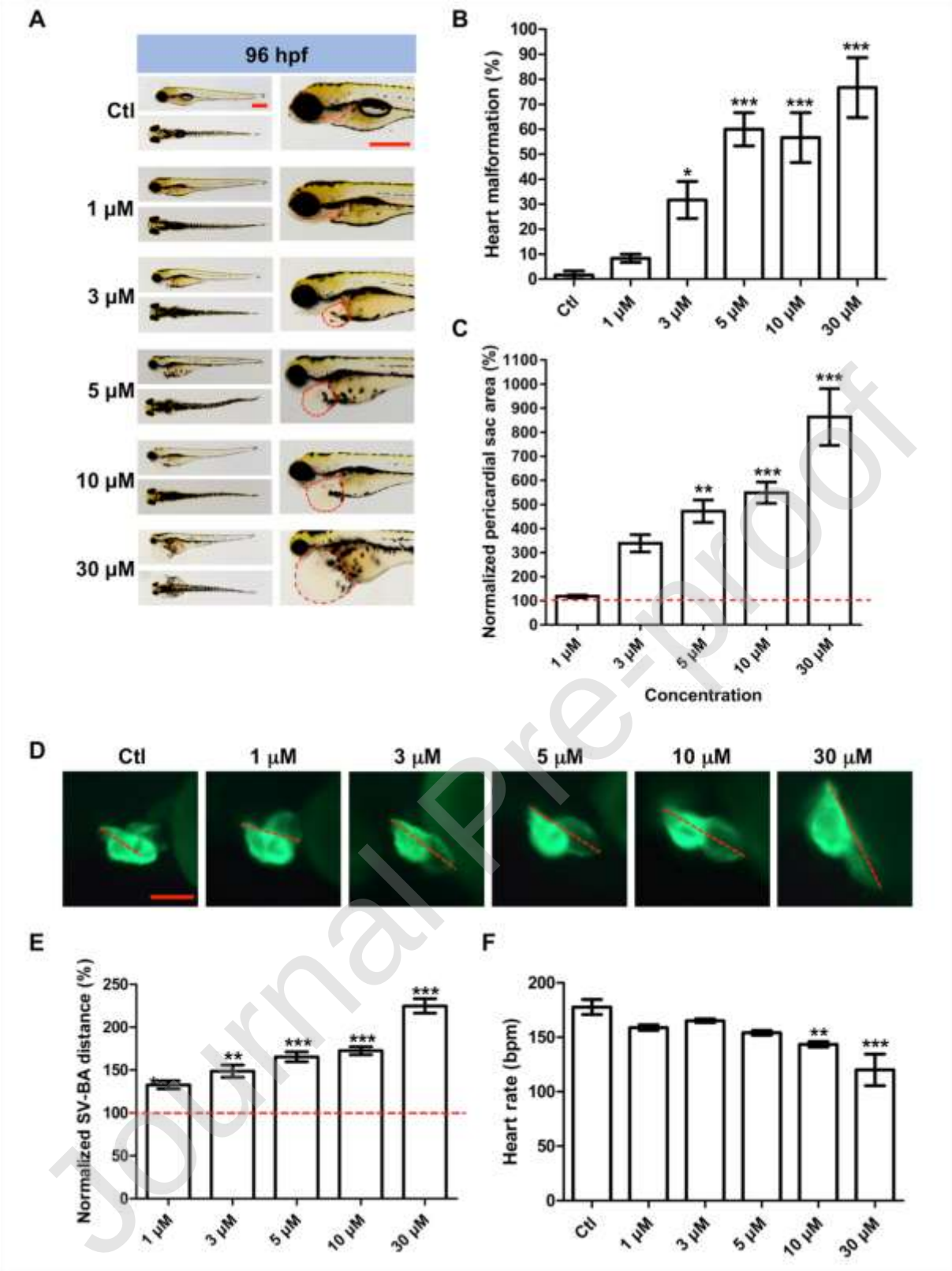

Figure 3. Cardiac defects after exposure to different concentrations of α-asarone. (A) Representative images of pericardial edema in zebrafish exposed to 1, 3, 5, 10, and 30 μM α-asarone at 96 hpf. Red dashed lines indicate the pericardium

area. (B) Heart malformation rate and (C) Normalized pericardial sac area of the zebrafish treated with different concentrations of α-asarone. (D) Representative images of *Tg (cmlc2: GFP)* zebrafish exposed to 1, 3, 5, 10, and 30 μM α-asarone at 96 hpf. SV-BA distance is indicated by red line. (E) Normalized SV-BA distance and (F) Heart rate of the zebrafish treated with α-asarone at 96 hpf. SV, sinus venous; BA, bulbus arteriosus. n=10 per group and the tests were repeated 3 times. Scale bar, 500 μm. *P < 0.05; **P < 0.01; ***P < 0.001 vs Ctl.

3.3 QT interval prolongation induced by α-asarone

To further investigate whether the cardiac function of zebrafish embryos was affected by α-asarone, ECG recording was performed. Consistent with other cardiotoxicity associated parameters tested in our study, α-asarone exposure caused abnormal ECG signals (Figure 4A). Specifically, as compared to a normal zebrafish ECG, a significant up-regulation in the mean QTc duration and smaller amplitude of T-wave were observed in the larval zebrafish treated with 30 μM of α-asarone (Figure 4B).

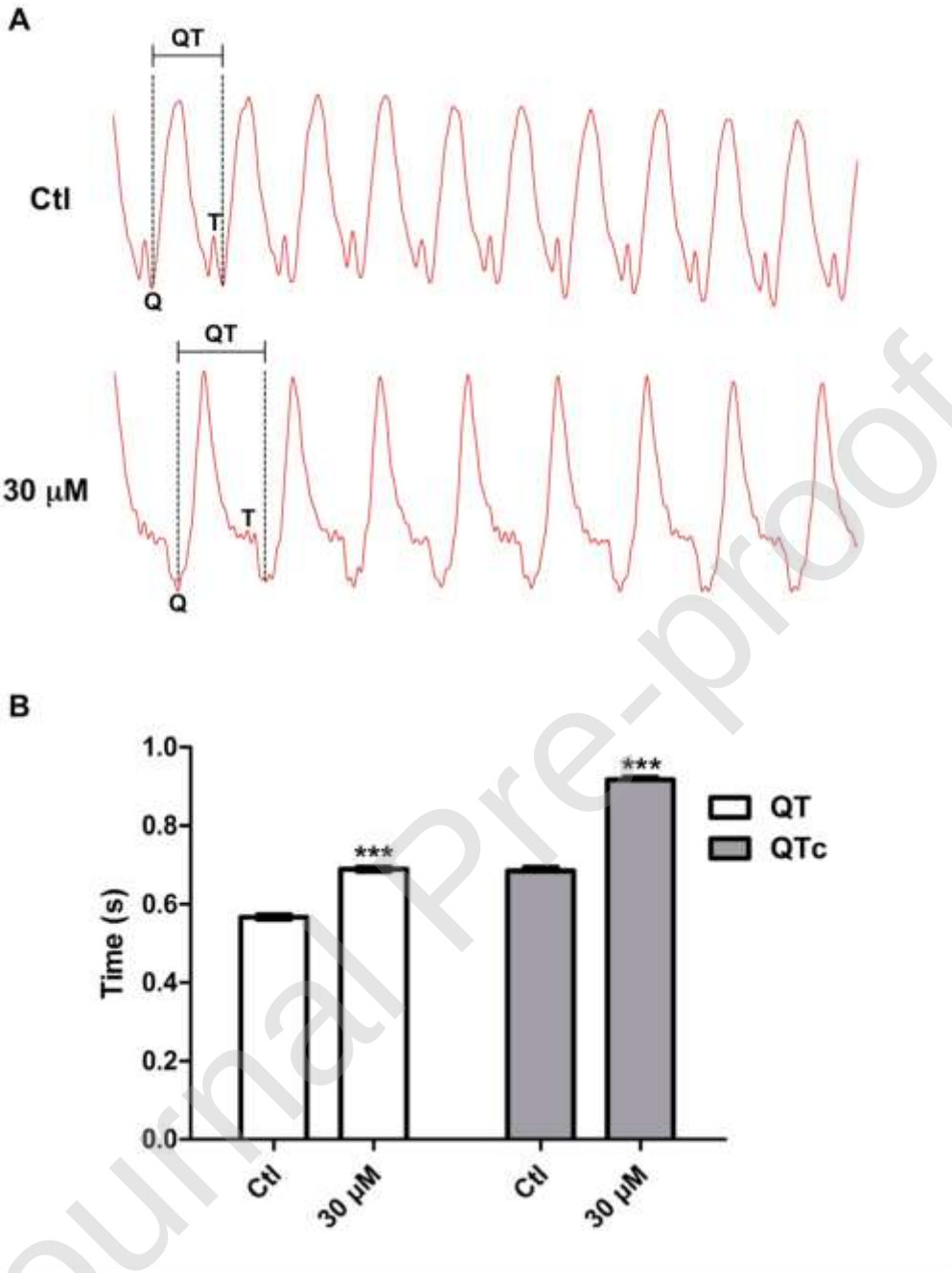

Figure 4. QT prolongation and T-wave abnormalities induced by α-asarone in zebrafish. (A) Representative images of the ECG waveforms in the control and α-asarone treated zebrafish. (B) QT interval and QTc interval. n=6 per group and the tests were repeated 3 times. ***P < 0.001 vs Ctl.

3.4 Gene expression of heart development-related key transcriptional regulators in α-asarone treated zebrafish

The mRNA expression of four heart development-related key transcriptional regulators was analyzed after treatment with α-asarone for 96 (Figure 5). The expression of *tbx5* and *nkx2.5* in α-asarone treated zebrafish was concentration-dependently increased compared with the control. For the mRNA levels of *hand2*, a significant down-regulation was observed in all groups exposed to α-asarone. The expression of *gata5* in the 30 μM group was markedly elevated compared with the control, while 1, 3, 5, and 10 μM of α-asarone exposure did not cause significant alteration.

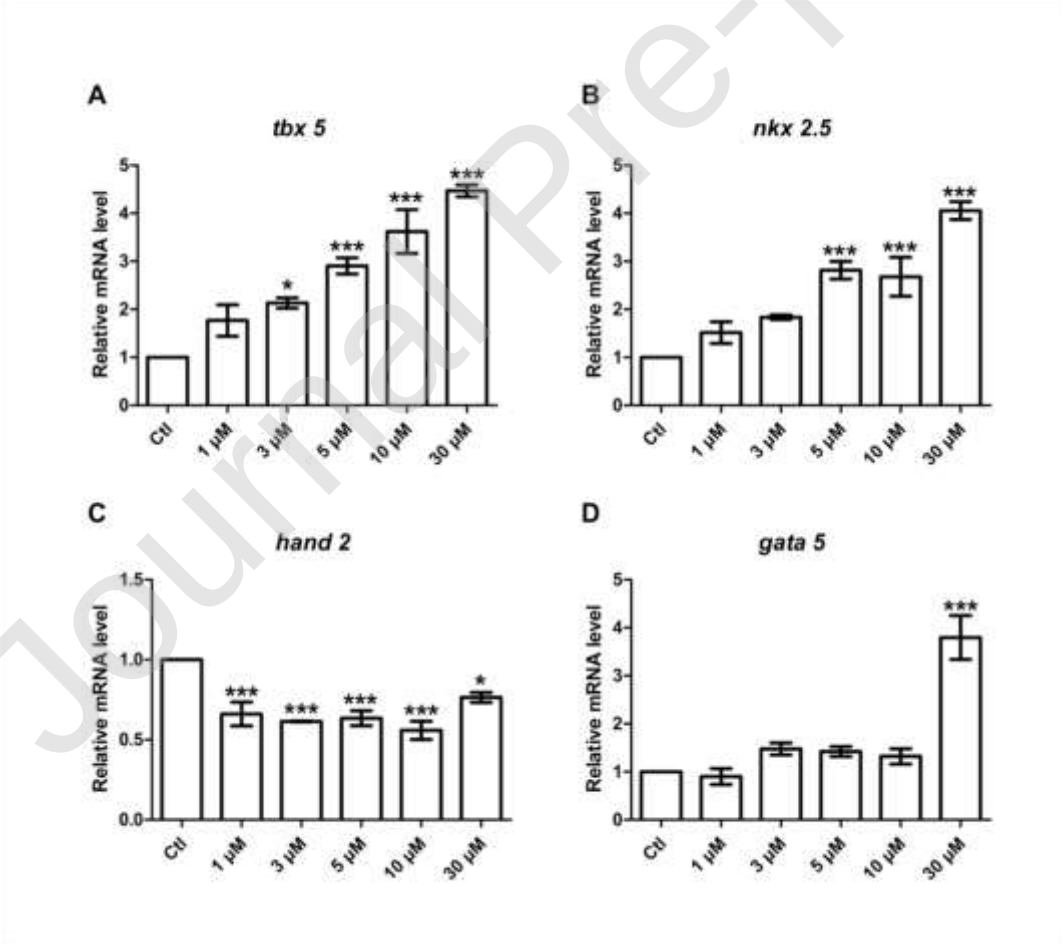

Figure 5. Expressions of heart development-related key transcriptional

regulators of zebrafish after exposure to different concentrations of α-asarone. (A-D) Relative mRNA levels of *tbx 5*, *nkx 2.5*, *hand 2*, and *gata 5*. The assays were repeated 3 times. *P < 0.05; ***P < 0.001 vs Ctl.

3.5 α-asarone triggered apoptosis mainly in the heart region of zebrafish

At 96 hpf considerable numbers of apoptotic cells were found in the heart region of zebrafish treated with α-asarone. In contrast, there was no obvious apoptotic cells in the control (Figure 6). Specifically, apoptotic cells stained with AO appeared mainly in the heart region after exposure to α-asarone for 96 hrs (Figure 6A and 6C). Higher number of apoptotic cells was observed with the increase in α-asarone concentration. Consistently, by using TUNEL assays, we found apparent apoptotic cells mainly in the heart area of zebrafish exposed to α-asarone in a concentration dependent manner (Figure 6B and 6D). Our apoptotic assessment implied that cardiac defects induced by α-asarone is likely due to apoptosis in the heart. Indeed, concentration-dependently increased expression of Caspase 3 was mainly detected in the heart region by immunohistochemistry on the whole-body cryostat sections of zebrafish treated with α-asarone (Figure 7).

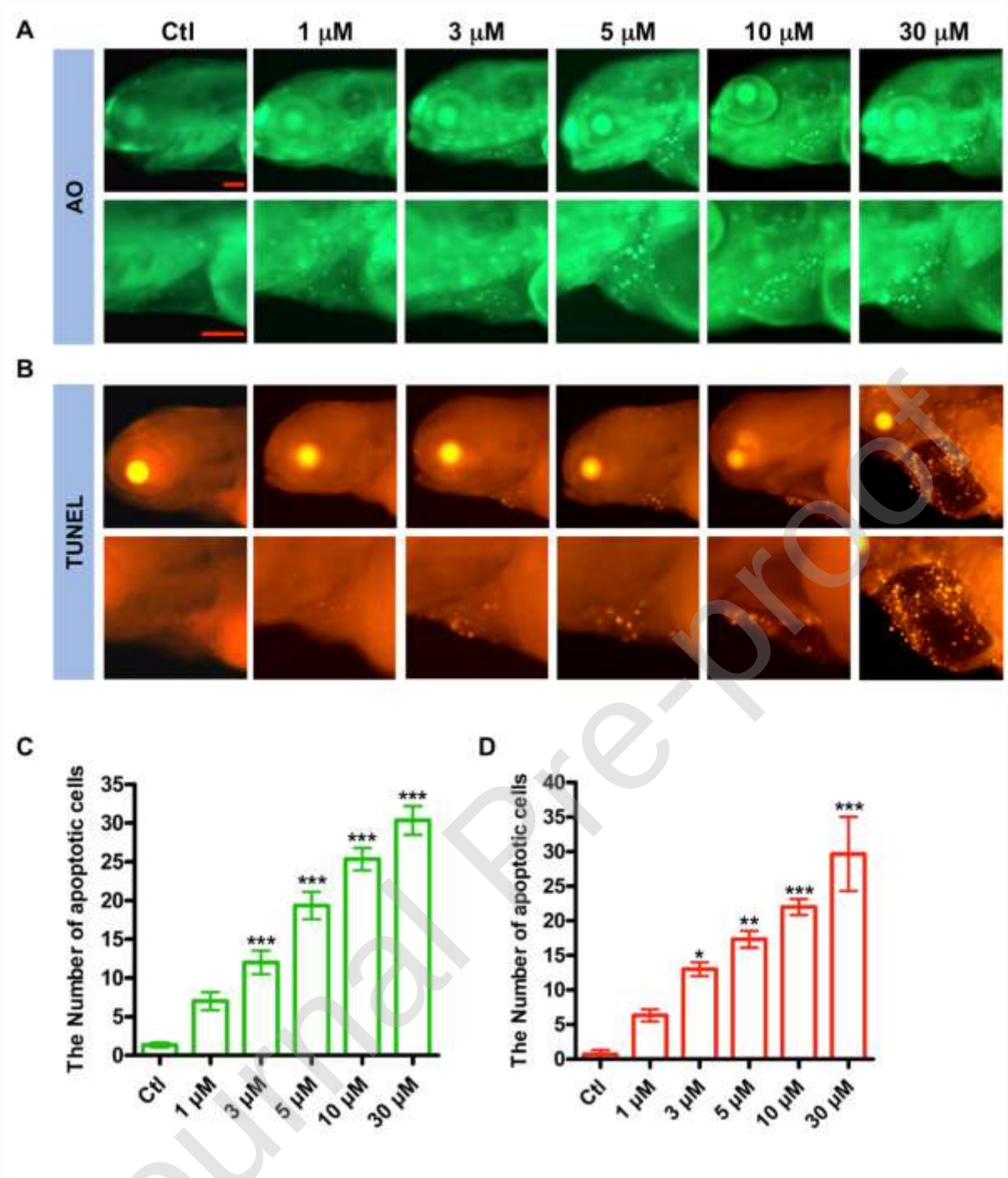

Figure 6. α-asarone-induced apoptosis in the heart region of zebrafish. (A-B) Representative images of the apoptosis of zebrafish exposed to 1, 3, 5, 10, and 30 μM α-asarone at 96 hpf stained with AO staining and TUNEL assay, respectively. (C-D) The number of apoptotic cells in AO staining and TUNEL assay, respectively. n=10 per group and the tests were repeated 3 times. Scale bar, 100 μm. *P < 0.05; **P <

0.01; ***P < 0.001 vs Ctl.

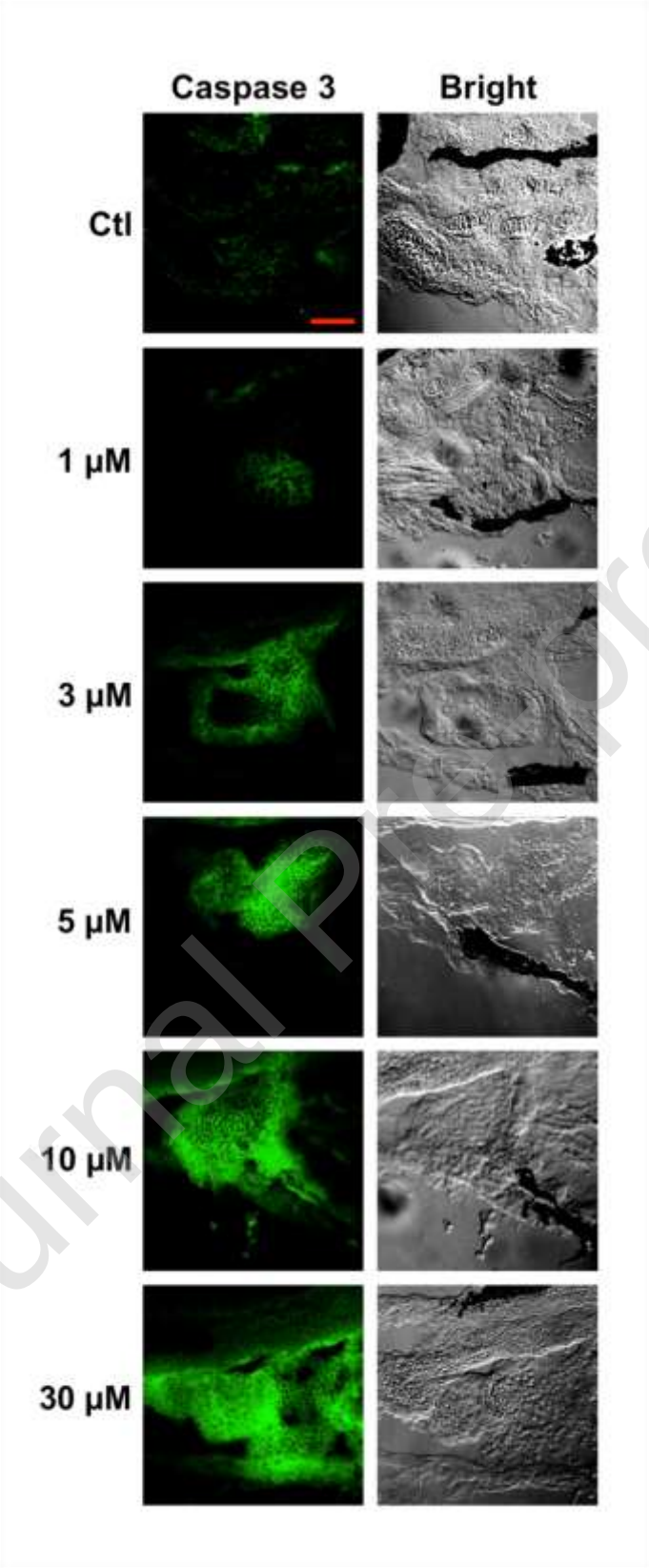

Figure 7. Caspase 3 expression in the heart of zebrafish after exposure to different concentrations of α-asarone. Representative images of Caspase 3

staining in the heart area of 96 hpf zebrafish treated with various concentration of α-asarone. Scale bar, 50 μm.

3.6 α-asarone induced mitochondrial apoptosis in the zebrafish heart

We then investigated the underlying mechanism of apoptosis. Using qPCR we found elevated expression of genes involved in mitochondrial apoptotic pathway in zebrafish treated with α-asarone (Figure 8). Specifically, we discovered an apparent up-regulation of *puma* and *cyto C* expression only in 30 μM group. 1 μM α-asarone treated zebrafish showed slightly higher mRNA expression of *afap*, *caspase 3*, and *caspase 9* as compared to the control, while their expression was dramatically increased in the zebrafish treated with 3, 5, 10, and 30 μM of α-asarone in a concentration-dependent manner. All these results suggested that mitochondrial apoptosis in the heart region of zebrafish is likely to be the main reason for α-asarone induced cardiac developmental toxicity.

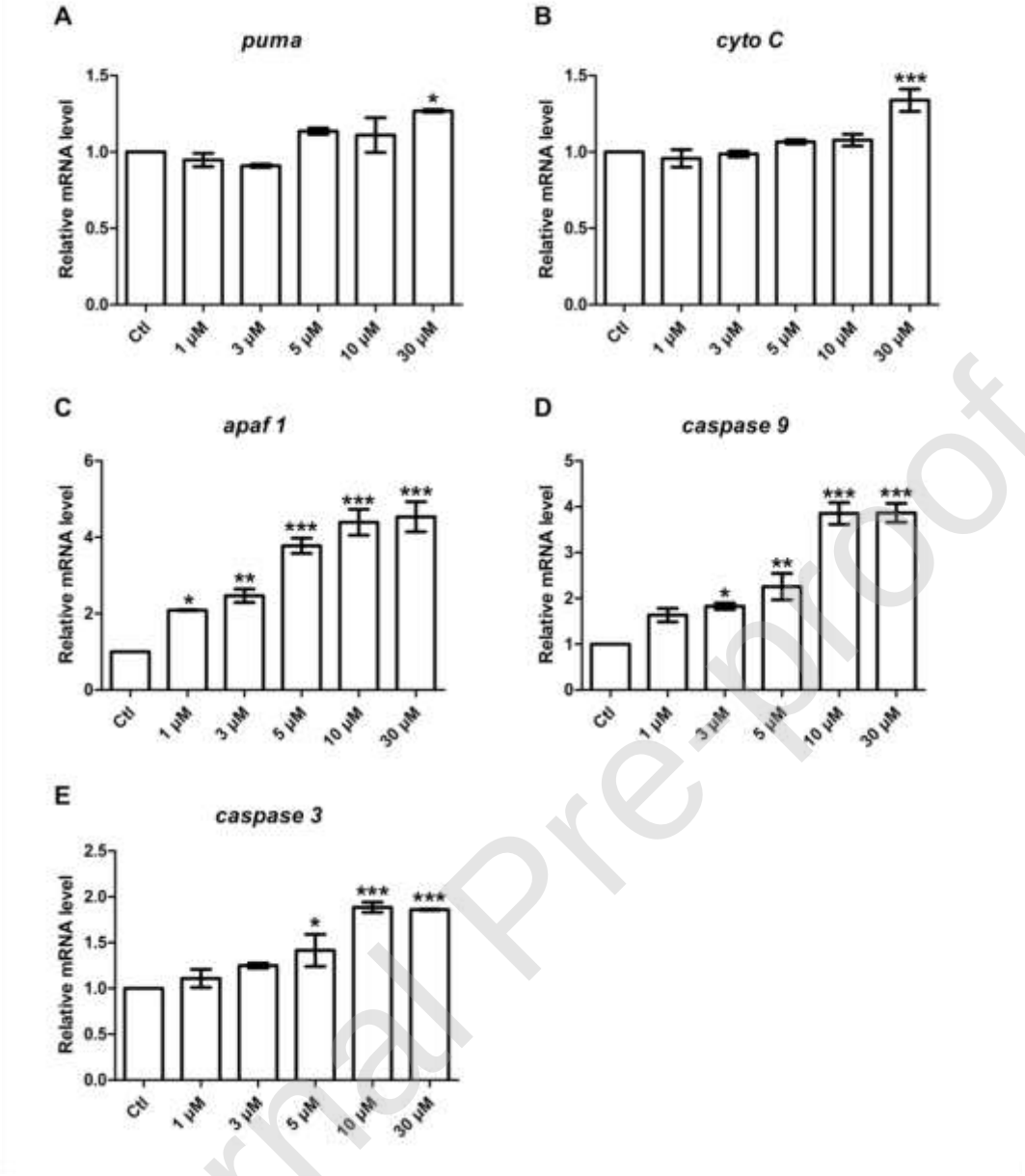

Figure 8. Expression of the genes involved in the mitochondrial apoptosis in α-asarone treated zebrafish. (A-E) The expressions of *puma*, *cyto C, afap1, caspase 9,* and *caspase 3* in zebrafish exposed to 1, 3, 5, 10, and 30 μM α-asarone. The assays were repeated 3 times. *P < 0.05; **P < 0.01; ***P < 0.001 vs Ctl.

4. Discussion

In this study, we showed the developmental and cardiac effects of α-asarone and sought to unravel the underlying link between its cardiotoxicity and the mitochondrial apoptosis. We found that: (1) α-asarone induces developmental and cardiac toxicity in zebrafish in a concentration-dependent manner; (2) α-asarone prolongs the mean QTc duration and causes smaller amplitude of T-wave; (3) Apoptosis is triggered in the heart region of α-asarone treated zebrafish and mitochondrial apoptosis is possibly involved in this process.

The US Food & Drug Administration forbade the use of *Acorus calamus* in beverages, pharmaceuticals, and dental preparations at 1974, in view of the animal studies indicating carcinogenic effects after its chronic oral administration [43]. Since α-asarone is the main components of *Acorus calamus*, the toxic effect α-asarone is worthy of attention. In the present study, the dose selection was mainly based on LC_1 (3.4 μM) and LC_{50} (32.7 μM) of α-asarone and the previous findings that 0.3-100 μM α-asarone administered to zebrafish embryos and primary microglia cells effectively modulates microglial morphological dynamics without any side effects [44]. Importantly, the daily intake dose of α-asarone in clinical use is approximately 115 μg [9], which is much higher than the dose used in our present study. Our data revealed that α-asarone at 1-10 μM has no significant influence on the hatching rate and mortality in zebrafish. This is consistent with the previous study that showed no developmental defects in zebrafish embryos treated with 0.3–3 μM α-asarone from 12 hpf to 5 dpf [44]. We found that only high concentration of α-asarone (30 μM) markedly induced mortality and malformation rate, while relatively low concentrations of α-asarone caused heart defects, implying that the heart is possibly the main target

for α-asarone causing toxicity. Furthermore, zebrafish treated with 30 μM of α-asarone showed decreased locomotor activity, which is in accordance with the report that α-asarone at a higher dose (200 mg/kg) significantly reduced spontaneous locomotor activity [45].

The heart, derived from the cardiac mesoderm, is the first organ formed during embryogenesis. Cardiac development is orchestrated by conserved cardiac transcription factors (TFs) [46, 47]. The core network of TFs and the main events that lead to heart formation is conserved in evolution. The cardiac TFs *tbx5, nkx2.5*, and *gata5* are pivotal to human heart development [48, 49]. Mutations and abnormal expression of these genes dramatically perturb heart development resulting in Congenital Heart Diseases (CHDs). Here, we found that α-asarone significantly changed cardiac development and function. Accordingly, zebrafish embryos exposed to α-asarone had up-regulated levels of *tbx5*, *nkx2.5*, and *gata5* as a compensatory response, as these genes regulate the cardiomyocyte differentiation [50], formation of the cardiac tube [51], embryonic cardiogenesis, and postnatal cardiac adaptive remodeling [52]. Previous studies in zebrafish uncovered essential roles of *tbx5* and *nkx2.5* during cardiac progenitor differentiation for maintaining ventricular and atrial chamber morphology as well as cellular traits later in development [53]. *gata5* is required for the migration of the cardiac primordia to the embryonic midline, its overexpression induces the ectopic expression of several myocardial genes including *nkx2.5* and can generate ectopic foci of beating myocardial tissue in zebrafish [54]. The increased expression of *nkx2.5*, *tbx5*, and *gata5* in α-asarone treated zebrafish is possibly due to regenerative capabilities of the zebrafish heart, augmenting the regenerative proliferation of cardiomyocytes in response to severe apoptosis-induced

injury. It has been reported that decreased expression of *hand2* resulted in fewer embryonic cardiomyocytes [55, 56], which is consistent with our findings that apoptosis was triggered by α-asarone in cardiomyocytes.

Mitochondria-dependent apoptotic pathway has a critical role in the regulation of cell survival or death [57]. The protein encoded by *puma* was found to be exclusively expressed in mitochondria [58]. Cytochrome c (cyto C) is a mitochondrial protein that triggers apoptosis in response to diverse stress inducers [59]. Cyto C induces caspase activation by binding the protein apaf1, resulting in activation of Caspase 9 [60]. After α-asarone treatment, we found an increase in the expression of caspase 3 and caspase 9, which are the key regulators required in the caspase cascade activation and execution [61]. We also found that the transcript levels of *puma*, *cyto C*, and *apaf1* were up-regulated in zebrafish treated with α-asarone, which suggested that α-asarone triggered apoptosis in zebrafish by activating mitochondria-dependent pathway, therefore causing cardiotoxicity (Figure 9). Similarly, previous studies indicated that β-asarone induced apoptosis in lung cancer cells, which was associated with the activation of Caspase 9 and Caspase 3, the up-regulation of Puma, as well as the translocation of Cyto C [62].

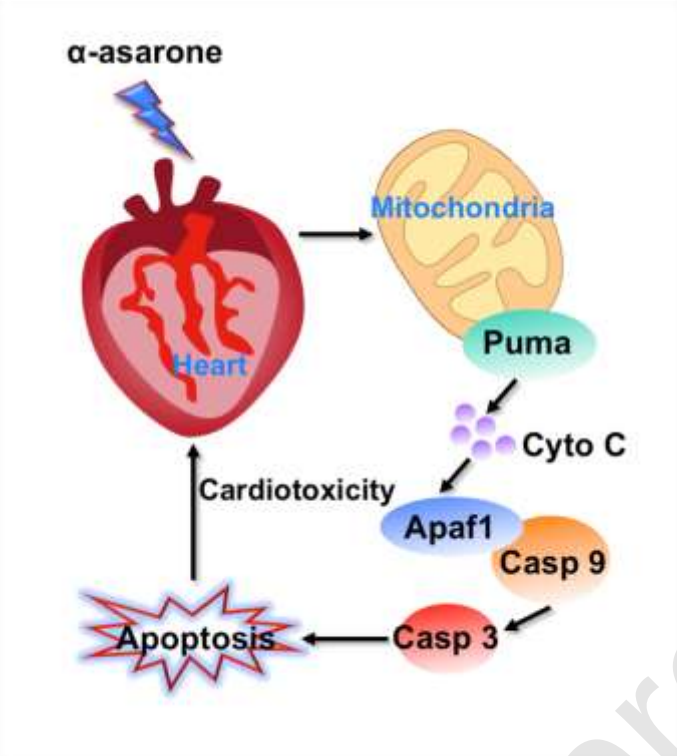

Figure 9. Schematic of mitochondrial apoptosis in α-asarone induced cardiotoxicity.

α-asarone triggers apoptosis by inducing Cyto C release from mitochondria into the cytosol. Apaf1 oligomerizes in response to Cyto C release and forms a large complex known as apoptosome. Caspase 9, an initiator caspase in the mitochondrial pathway, is recruited and activated by the apoptosome. Activated Caspase 9 cleaves pro-Caspase 3 to form active Caspase 3, thereby induce apoptosis in cardiomyocytes.

5. Conclusion

Our study provides a clear evidence that α-asarone produces cardiotoxicity in zebrafish. Mitochondrial apoptosis in the heart region is likely to be the main reason for this process. Considering α-asarone-induced toxic effect in cardio development, caution has to be taken when determining the dose and duration of α-asarone usage

in order to avoid side effects.

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Author Contributions: MJ, XLS, and KCL conceived the project and designed the experiments. MJ, XNJ, and JD performed the experiments and analyzed the data. AS, LZW, and CS provided expertise on data analysis. MJ and XLS wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Declaration of interests

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.

Supplementary materials:

Table S1. Real-time Quantitative PCR primer sequences.

Figure S1. Effect of α-asarone on locomotion in zebrafish. (A) Total distance traveled of larval zebrafish exposed to α-asarone for 120 hrs. The distance moved for each larva from each group was analyzed using Zeblab software. n = 12 per group. (B) The average speed of the larval zebrafish from different groups. ***P<0.001 vs Ctl.

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