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Toxicology of milrinone by zebrafish embryotoxicity test

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Abstract

Milrinone, a phosphodiesterase III inhibitor with contractile and vasodilatory effects, is widely used in acute decompensated heart failure and medically refractory end-stage heart failure (HF). The adverse reactions of milrinone have been extensively explored clinically, but its possible toxicities and underlying molecular mechanisms in embryo development need further understanding as its clinical applications increase. Herein, we assessed the milrinone toxicity using the zebrafish embryotoxicity test (ZET), with a view of providing evidence and guidance for gravidas medicine. We carried out ZET by exposing embryos to a milrinone culture with a series concentration gradients since 1.5 hours post fertilization (hpf) and observed and assessed mortality and hatching rates of drug-treated zebrafishes at 24, 48, 72, and 96 hpf. No significant lethal effect was found in milrinone-treated zebrafish, but hatching rate of eggs at 48 hpf was up-regulated with the increase of milrinone concentration. The impact of milrinone on embryogenesis was assessed through body length, eye area, yolk sac area, swim bladder inflation area, pericardial area and venous congestion area at 96hpf. 150 μg/mL or higher milrinone treatment showed significant effects in the indicators. Organ disorders including enlarged pericardium, liver atrophy and decreased blood vessels were observed in dysplasia individuals versus controls. TUNEL assay suggested the ability of milrinone to induce apoptosis in malformation embryos. Quantitative real-time PCR showed aberrant expressions of transcription factors associated with heart development and genes related to liver development and apoptosis regulation. Therefore, ZET is feasible for the milrinone toxicity test, and high-dose milrinone causes harm to the embryonic development of zebrafish, especially in embryonic carcinogenesis, vasculogenesis, and hepatogenesis.

Keywords: Milrinone, Pericardial area, Vasculogenesis, Hepatogenesis, Quantitative real-time PCR

1. Introduction

Cardiogenic shock (CS) is featured by cardiac pump failure-induced systemic hypoxia and end-organ hypoperfusion. Addressing hemodynamic abnormalities at an early stage can relieve multisystem organ failure progression and lower mortality. Dobutamine and milrinone are the most extensively prescribed inodilators in the CS clinical trials [1].

Milrinone, a homolog of amrinone, is a phosphodiesterase III inhibitor (PDE-III). It's clinically applied to intractable congestive heart failure (CHF), cardiomyopathy, low cardiac output syndrome (LOS) after cardiac surgery, acute HF after cardiac surgery, etc. [2,3]. Milrinone has both positive inotropic and peripheral vasodilation effects and can improve diastolic ventricular compliance. Its pharmacological mechanism is to inhibit the activity of PDE-III, reduce the inactivation of cyclic adenosine monophosphate (CAMP) in cardiac myocytes, elevate cyclic CAMP concentration in cardiac myocytes, enhance intracellular calcium, strengthen myocardial contractility, and produce positive muscle force. Meanwhile, it has a positive relaxation effect on vascular smooth muscle, which can expand blood vessels to lower cardiac preload and afterload, thus reducing left ventricular filling pressure, enhancing left ventricular function, and increasing cardiac index without increasing myocardial oxygen consumption [4-7].

Previously, it has been reported that the adverse reactions of milrinone including headache, arrhythmia, worsening of muscle weakness, and decreased platelet count were found in clinical patients [8-10]. However, clinical trials abroad confirmed that long-term oral milrinone can increase the mortality of patients with HF from non-HF causes [11-13]. Besides, it remains to define the safe medication dose range, toxicology, and molecular mechanism of toxicity of milrinone. Considering the toxicity of milrinone, it should be used with caution in clinical use in pregnant and lactating women.

To directly determine milrinone's toxicity mechanism in embryogenetic stage, we performed zebrafish embryotoxicity test (ZET) by milrinone exposure with a range of concentrations, and found the dose-dependent adverse effects in zebrafish embryos, suggesting its feasibility for

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clinical use in gravidas.

2. Materials and methods

2.1. Strains and culture of zebrafishes

Wild-type zebrafish of the following stains were supplied by China Zebrafish Resource Center: AB genotype (Catalog ID: CZ1), *gz15Tg*/+(AB) transgenic zebrafish line (Catalog ID: CZ 16), *y1Tg*/+(AB) transgenic zebrafish line (Catalog ID: CZ55), and *fl1Tg*/+(AB) transgenic zebrafish line (Catalog ID: CZ 56). The *gz15Tg* allele (CZ 16) is a bicolor transgenic line *Tg (fabp10a:dsRed;ela3l:EGFP)* that co-expressesed DsRed RFP in the liver and EGFP in the exocrine pancreas. The mutation *y1Tg* (CZ 55) is generated by random integration of a GFP-containing construct, predominantly expresses GFP in cranial neural crest derivatives and blood vessels. GFP expression can be detected at low levels as early as 3-somite stage. *fl1Tg* allele genotyping revealed a transgenic zebrafish line (CZ 56) with EGFP signals on the cardiomyocyte plasma membrane. While green fluorescence was found in periventricular and atrioventricular duct cardiomyocytes in specific transgenic zebrafishes. Transgenic strain CZ 56 can be utilized to determine zebrafish embryonic distance between venous sinus (SV) and arterial bulb (BA), and CZ 16 and CZ 55 to measure the shape and size of the liver and vessels.

In the 28.5°C water, they were raised in pair-mating tanks with a female-to-male ratio of 1:2 for egg production under a standard light/dark (14/10 h) cycle that began at 8:10 am. An oviposition dish was inserted into each tank at the beginning of the photoperiod and left for 30 minutes before the eggs were collected. Eggs of each hybrid group were collected, washed and transferred to the Petri dish, for a 45-minute incubation in a 28.5°C incubator. The fertilization rate of each hybrid group was then determined by randomly selecting 30 embryos. Three groups of fish eggs were mixed for subsequent studies.

2.2. Embryo exposure to milrinone and embryonic cell apoptotic assay

Phenotypic statistical experiments were performed on wild-type zebrafishes (CZ1). Their embryos and juveniles were first immersed in an E3 medium. A series concentrations of milrinone [Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China, Catalog ID: 78415-72-2, ≥98% (HPLC)], namely, 0.8, 4, 20, 100, and 200 μg/mL, were then prepared by diluting with the E3 medium, with a 0 μg/mL milrinone medium as a control. After grouping, the zebrafish eggs were transferred into the wells of 6-well plates at 1.5 hours post fertilization (hpf). Each group was then grouped into three subsamples of 30 eggs per well for incubation in a milrinone medium with a given concentration. Medium change (4 mL corresponding fresh milrinone dilution) was performed on a daily basis. We recorded and analyzed the mortality and hatching rate of each subsample at 24 hpf, 48 hpf, 72 hpf and 96 hpf, and measured embryo body length, eye area, yolk sac area, swim bladder inflation area, pericardial area and venous congestion area after milrinone exposure at 0, 100, 150, and 200 μg/mL for 96 hpf using the Capture 2.0 software (Nikon, Tokyo, Japan, SMZ800N).

Cell apoptosis in the previously measured zebrafishes at 96hpf was then determined as instructed by the One-step TUNEL assay kit (Beyotime Biotechnology, Shanghai, China, Cat NO: C1086) recommendations. This was followed by the fixation of all zebrafish samples and cultivation in a TUNEL mixture containing Acridine Orange (AO)—a nucleic acid-binding dye that fluoresces green when it is bound to 3 '- OH end of broken dsDNA. Those stained green in zebrafish embryos were apoptotic cells. Then, we selected a specific view field to count apoptotic and non-apoptotic regions microscopically, with the apoptotic index indicated by the green fluorescence intensity, followed by image analysis with the Image J software (URL: https://imagej.en.softonic.com/).

2.3. Behavioral acquisition

At 120 hpf, the AB strain larvae with milrinone treated were planted onto 96-square-well plates (one larva/well) for a 5-minute warming in the 28°C incubator, followed by transfer to a Daniovision® chamber (Noldus Info Tech., Wageningen, The Netherlands). Larvae were acclimatised for 30 min prior to the test, and then video recording was started. The visible light was turned off a quarter after recording and turned back on 45 min later. The recording continued for another three-quarters of an hour, for 1 h 45 min in total. The EthovisionXT software purchased from Noldus Info Tech., Wageningen, The Netherlands was utilized for trajectory analysis, and data on velocity were exported to Excel and visualized with GraphPad Prism 7. Locomotor activity was recorded as the average distance travelled per second (mm/s) over 50 min, and the total distance travelled (mm) during the test period was statistically analyzed. Data were calculated for 10 larvae that received each treatment.

2.4. Milrinone toxicology test

CZ56 zebrafish embryos were grouped and seeded onto 6-well plates for incubation with milrinone medium (4 mL) at 0, 100, 150, and 200 μg/mL at 1.5 hpf, respectively. Three replicates were set up, with 30 embryos per treatment. Culture medium replacement with 4 mL of corresponding fresh milrinone dilution was carried out once every 24 hours. We recorded the embryo heart rate (HR) at 96 hpf, and measured the distance between venous sinus (SV) and arterial bulb (BA) by detecting the green fluorescent protein (EGFP) expression in the whole cardiomyocytes of zebrafishes.

CZ16 and CZ55 strain zebrafishes were tested in the same way as the above-mentioned CZ56 strain. We measured the red fluorescence area to determine the CZ16 strain zebrafish liver size, the Capture 2.0 software to calculate the EGFP area in CZ56 strain and RFP in CZ16 strain, and the GFP to label and count the CZ55 blood vessel count.

2.5. Quantitative real-time PCR (Q-RT PCR) in Zebrafish embryos

Q-RT PCR was carried out on the total RNA isolated from zebrafish embryos with RNAiso Plus (TAKARA, Cat NO: 9109) after milrinone exposure at 0, 100, 150, and 200 μg/mL for 48 hpf respectively. The Novoprotein Reverse Transcription Kit (Cat NO: abs60077) was employed for cDNA synthesis. Primer pairs presented in Table 1 were utilized for gene expression analysis of sox9b, NKX2.5, GATA4, P53, BAX, Caspase-3, Caspase-9, bcl-2, GCLC, Apaf1, ef1a, CDH5, kdrl, Prkl and Fabp10a relative to β-actin gene. The Q-RT PCR was performed using an Archimed™ X4 medical fluorescence quantitative PCR instrument (RocGene Technology Co., Ltd, China)

Table 1. Oligos used for Q-RT PCR.

Gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
sox9b	AGACGCAGATCTCCACCAAT	CAGATCCGCTTTACTGCACA
NKX2.5	GTTATTTAGATCCCCAACACCA	AACACCTACCCTGCGTTTAGT
GATA4	ACCACCTCCTATAACTCAAGCACC	CAGAGCCCGAGACCCGAAAT
P53	GGGCAGGGAGCGTTATGA	AGAGTCGCTTCTTCCTTCGTC
BAX	GGCTATTTCAACCAGGGTTCC	TGCGAATCACCAATGCTGT
Caspase-3	TCGCAGGACAGGCATGAAC	GTGATCGTCATGGGCAACTG
Caspase-9	TTCTTCAGCGGCACAGGTTA	GTCTGGTTGCCTTGCTCTGTA
bcl-2	ATGTGCGTGGAAAGCGTCAAC	GAAGGCATCCCAACCTCCATT
GCLC	AAAATGTCCGGAACTGATCG	AACGTTTCCATTTTCGTTGC
Apaf1	TTCCGTCAGCGGTGTAAGGT	TTACAATGCTGCGGGCCTGT
ef1a	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC
CDH5	ACTGCATGTCCTACGCAAGG	ATAGCGCCGTCTCAACACAA
kdrl	ACTGCATGTCCTACGCAAGG	ATAGCGCCGTCTCAACACAA
Prkl	TCCGCAATTTCGAGCAGGTC	GCAGAATTGCAGCGTCCGAT
Fabp10a	CTCCTTCACCATCGGCAAAG	TCTCCAGCCTTGATCTCCTG
β-Actin	TGAATCCCAAAGCCAACAGA	GGAAGAGCGTAACCCTCATAGA

with Novoprotein Real-time Fluorescence Quantitative PCR Kit (Cat NO: E096). Gene expression relative quantification was analysed based on $2^{-\Delta\Delta CT}$ [14], which displayed the fold change relative to the control after normalization against β-actin. Over 3 independent replicates were carried out for each experimental treatment.

2.6. Statistical analysis

This study employed GraphPad Prism 7.0 (La Jolla, CA, USA) for statistical analyses. Statistical inter-group differences (threshold: $P < 0.05$) of statistic parameters, given mean±standard deviation (SD), were identified by one-way ANOVA. * indicates $P < 0.05$; **, $P < 0.01$; ***, $P<0.001$; ****, $P<0.0001$; ns, not significant.

3. Results

3.1. Milrinone Toxicity Evaluation of the Developmental Zebrafish Embryos

To determine milrinone's toxicity to zebrafish embryos, we observed embryos after long-term milrinone administration. No lethal effect was observed in zebrafish embryos with milrinone doses from 0.5 to 200 μg/mL (Data were not shown). The increasing milrinone concentration ware significantly accelerated the hatching rate of embryos at 48 hpf (Figure 1A). How various milrinone concentrations influenced embryogenesis was then examined. Morphological indices like body length, eye area, yolk sac area, swim bladder inflation area, pericardial area and venous congestion area were measured at 96 hpf. Significantly reduced eye area and body length of the embryos were observed following 150 and 200 μg/mL milrinone treatment (Figure 1B-E). While 200 μg/mL milrinone treatment resulted in markedly increased yolk sac area of embryos compared to the control (Figure 1F). Delayed yolk sac absorption is a typical drug exposure-induced embryonic developmental toxicity. Besides, failed swim bladder inflation was found after milrinone intervention (Figure 1G). Augmentation of the pericardial area and venous congestion area were found in 150 and 200 μg/mL milrinone-treated embryos and suggested the cardiotoxicity of milrinone (Figure 1H-I).

3.2. Toxicity of Milrinone to zebrafish cardiac development

For cardiotoxicity determination, we measured the distance between venous sinus (SV) and arterial bulb (BA),

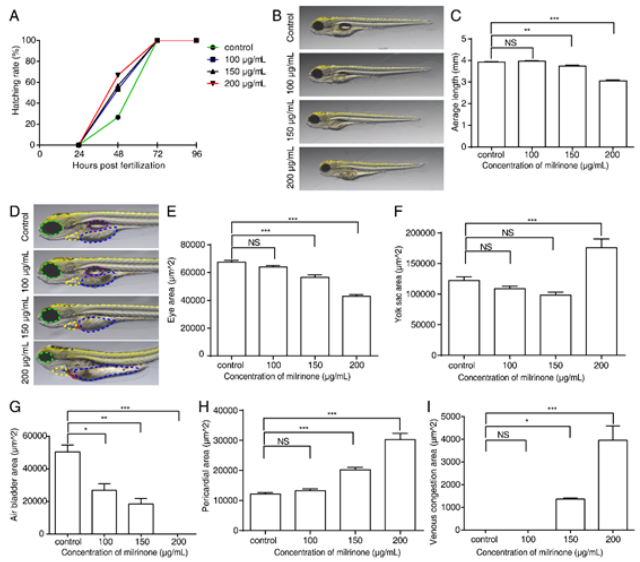

Fig. 1. Developmental toxicities in zebrafish embryos after embryonic exposure to series milrinone concentrations. (A) The milrinone increased the hatching rate of zebrafish eggs to 48 hpf. Milrinone was nonlethal toxicity toxic to zebrafish embryos, The hatching rate had no difference between milrinone-treated eggs and control at 24 hpf, 48 hpf and 96 hpf, respectively. (B-C) Representative pictures (B) of zebrafish after 0, 100, 150, and 200 μg/mL milrinone exposure at 96 hpf, and the average body length column statistics (C) of zebrafish with the corresponding milrinone treatment. (D) Representative statistical images for the developmental toxicity analysis of milrinone-treated zebrafish. Zebrafish eye, yolk sac, air bladder, pericardium and venous congestion were respectively signed with green, blue, pink, yellow and red dotted lines marking. (E-I) The eye area (E), yolk sac area (F), air bladder area (G) pericardial area (H) and venous congestion area (I) of zebrafish embryos were analysed after milrinone exposure at 0, 100, 150 and 200 μg/mL for 96 hpf, respectively. Mean ± SEM; n = 30, repeat for three times. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

and zebrafish embryo HR and blood vessels for the study of cardiovascular physiological function. Increased SV-BA distance was found in CZ56 zebrafish larva with 200 μg/mL milrinone at 96 hpf (Figure 2A-B). Besides, we observed slightly up-regulated HRs of embryos intervened by 200 μg/mL milrinone at 48 hpf (Figure 2C). A significant decrease of blood vessels in milrinone-exposed zebrafish embryos at 100, 150, and 200 μg/mL versus the control were determined at 96 hpf (Figure 2D-E).

HR variability, which is regulated by NKX2.5, is a known important clinical marker [15]. Constantly, NKX2.5 rose in 200 μg/mL milrinone-intervened zebrafishes (Figure 2F). In addition, sox9b and GATA4, transcription factors associated with zebrafish cardiac anomalies, altered evidently in specific milrinone-treated zebrafishes. Vegf-a binds and activates kinase insert domain receptors like (kdrl), which enables protein tyrosine kinase activity to participate in angiogenesis and peptidyl-tyrosine phosphorylation in zebrafishes [16]. Figure 2F shows that milrinone could significantly decrease mRNA levels of *kdrl* in zebrafish after drug exposure at 100, 150, and 200 μg/mL for 48 hpf, it suggested that the impact of milrinone on the angiogenesis caused the decrease of zebrafish blood vessels (Figure 2E). Cadherin 5 (CDH5), or vascular endothelial cadherin, is the major cadherin in endotheliocytes. CDH5 is activated in glioblastoma stemlike cells and contributes to formation of blood vessels induced by hypoxia [17]. Here we found that the expression of CDH5 was upregulated in zebrafish after drug exposure at 150 μg/mL for 48 hpf (Figure 2F).

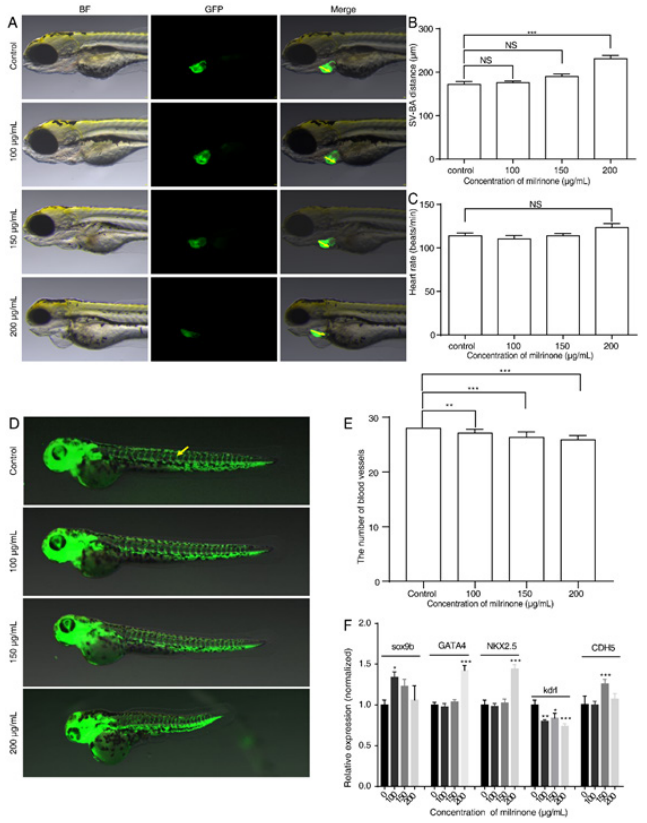

Fig. 2. The cardiovascular system dysplasia in zebrafish was driven by milrinone treatment. (A) The representative images of CZ 56 Zebrafish after milrinone (0, 100, 150, and 200 μg/mL) exposure for 96 hpf. The EGFP fluorescence was expressed in cardiomyocyte plasma membrane. (B-C) SV-BA distance lengthening (B) indicated by the yellow arrow was significantly increased in zebrafish after 200μg/mL milrinone exposure at 96 hpf, but the heart rate (C) was aroused slightly but insignificantly in zebrafish treated with 200 μg/mL milrinone 96 hpf. Mean ± SEM; n= 10. ***P < 0.001; NS, not significant; 0 μg/mL milrinone treated group used as control. (D) The CZ55 Zebrafish was captured after milrinone exposure at 0, 100, 150, and 200 μg/mL for 48 hpf, respectively. The GFP fluorescence was predominantly expressed in cranial neural crest derivatives and blood vessels (yellow arrow). (E) The number of blood vessels was decreased accompanied by increasing concentration of milrinone exposure in zebrafish at 48 hpf. (F) Milrinone-intervened zebrafishes showed up-regulated expression of Sox9b (a cardiac development-associated transcription factor) at 48 hpf. Similar effects were observed in GATA4, NKX2.5 and DH5 gene levels. Conversely, kdrl decreased in larvae intervened by 100, 150, and 200 μg/mL milrinone. Analysis was performed using the Student-t test. Error bars represent the SD. *P < 0.05; **P < 0.01; ***P<0.001; NS, not significant.

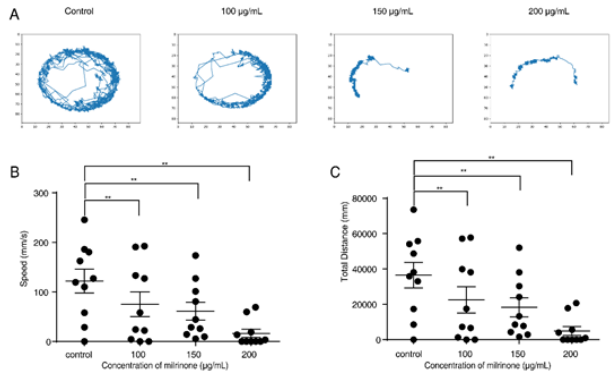

Fig. 3. Milrinone intervention induced locomotion degeneration in zebrafish larvae. (A) Representative images of milrinone (0, 100, 150, and 200 μg/mL)-exposed larvae behavior trajectory at 96 hpf. The collection method based on EthovisionXT software. (B-C) The mean distance moved per minute over 50 minutes (B) and the total distance travelled during the test period (C) statistically analyzed. Data were calculated for 10 larvae that received each treatment. Mean ± SEM; n= 10. **P < 0.01.

In clinical trials, the worsening of muscle weakness side effects were reported in patients with milrinone exposure [18], hence the zebrafish behavior observation system was introduced to automatically track and quantify the zebrafish behavior during milrinone administration (Figure 3A). The experimental data revealed that swim speed (Figure 3B) and total distance travelled (Figure 3C) were significantly decreased in zebrafish after drug exposure at 100, 150, and 200 μg/mL, respectively.

3.3. Toxicity of Milrinone to the liver of zebrafishes

A reporter Zebrafish line with *gz15Tg* allele specifically expressing DsRed RFP in liver was constructed. RFP began to express in the Zebrafish liver at 48–53 hpf and was employed for liver physiological status assessment. Milrinone (150 and 200 μg/mL)-intervened zebrafish larvae exhibited liver atrophy at 96 hpf, which was worsened by the increasing milrinone concentration (Figure 4A-B). According to Q-RT PCR, there was a connection between liver development-related gene (Fab10 and VTG) expression and milrinone dosage. Obviously reduced Fab10 expression was detected in embryos intervened by 100, 150, and 200 μg/mL of milrinone at 96 hpf. Similar down-regulated patterns of VTG expression among 150 and 200 μg/mL milrinone-intervened embryos were confirmed, although

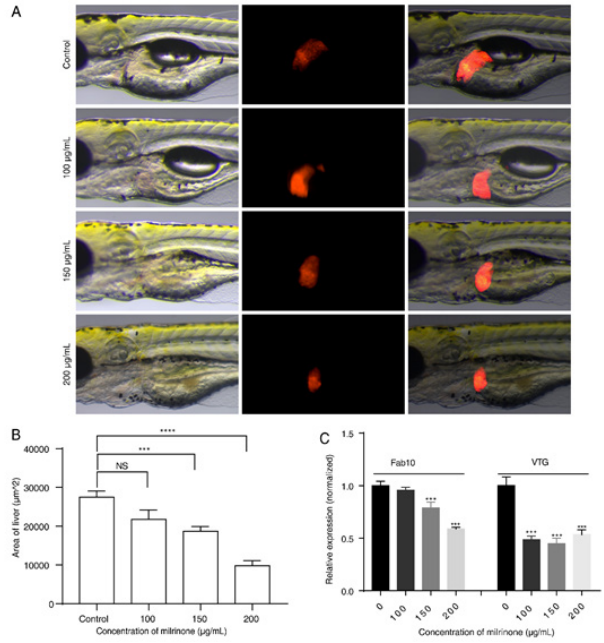

Fig. 4. Milrinone intervention triggered liver dysfunction in zebrafish larvae. (A) Typical images of milrinone (0, 100, 150, and 200 μg/mL)-exposed CZ 16 zebrafishes captured by fluorescence microscope at 96 hpf. DsRed RFP was expressed in the liver. (B) A high dosage of milrinone application induced severe liver atrophy in liver malformation individuals at 96 hpf. 0 μg/mL milrinone treated group used as control. 2-way ANOVA was performed. Mean ± SEM; n= 10, ***P < 0.001; ****P < 0.0001. (C) 150 μg/mL and 200 μg/mL milrinone-intervened zebrafishes showed down-regulated expression of Fab10 (a cardiac development-related transcription factor) at 96 hpf. A similar effect was observed in VTG gene expression. Student-t test was performed. Error bars represent the SD. ***P<0.001; NS, not significant.

not found in 100 μg/mL milrinone-intervened embryos (Figure 4C).

3.4. Toxicity of Milrinone to apoptosis in zebrafish embryos

To cellularly clarify the role played by milrinone in Zebrafish embryos, apoptosis of milrinone-intervened embryos at 96 hpf was analyzed using the TUNEL method under fluorescent microscopy. Treatment with milrinone-induced cell apoptosis in embryos (Figure 5A-B). Apoptotic peptidase activating factor 1 (apaf1), enabling cysteine-type endopeptidase activator activity, participates in the intrinsic apoptotic axis in response to endoplasmic reticulum stress. The mRNA level of apaf1 was dramatically increased in embryos with milrinone treatment at 96hpf. p53, an apoptosis regulator, presented evidently elevated expression in 150 and 200 μg/mL milrinone-intervened embryos. Like p53, Bcl2 expression level went up in embryos with 100, 150, and 200 μg/mL milrinone intervention. Consistent caspase3 expression patterns in 100 μg/mL milrinone-treated embryos were presented (Figure 5C).

4. Discussion

Milrinone, also known as bipyridine ketone, methyl cyanide topiramate ketone, is original developed for the treatment of HF [19]. Previous clinical trials to judge the effect of milrinone in HF revealed few side effects such as headache, arrhythmia, weakness, and decreased platelet count. However, the elevated risk of arrhythmias and hypotension can be alleviated by limiting the milrinone dose used [20]. Moreover, more than six months long-term milrinone administration can increase the mortality of patients with HF from non-HF causes. Nevertheless, little is known regarding milrinone-induced adverse events in pregnant and lactating women. Herein, we conducted experiments on zebrafish embryos to assess milrinone toxicity, and investigated the connection between administered doses and adverse events, rendering clinical guidance for the milrinone use in pregnant women.

4.1. Milrinone toxicity to zebrafish embryo development

This study employed ZET to determine milrinone toxicology, wherein a series of milrinone concentrations including 0, 0.5, 20, 100, 150, and 200 μg/mL were used. We observed 200 μg/mL milrinone-induced adverse effects in embryos, ranging from body length shortening, eye area reduction, yolk sac area expansion, and postponement of swim bladder inflation to dilated pericardium and venous congestion area increasing. Our finding also underscored cardiac, vascular, and hepatic histological variations.

4.2. Cardiotoxicity and hepatotoxicity of milrinone to Zebrafish Embryos

Cardiovascular physiological functions in humans and zebrafishes are conserved anatomically and even cellularly. Many human cardiovascular agents have shown similar effects on zebrafishes [21]. Our findings have revealed the occurrence of pericardial dilatation, SV-BA distance lengthening and other adverse events in zebrafish embryos by 200 μg/mL milrinone. Moreover, 200 μg/mL milrinone exposure had slightly increased the heat rate, although it has no significant difference compared to the control; but these results agree with research in human and cynomol-

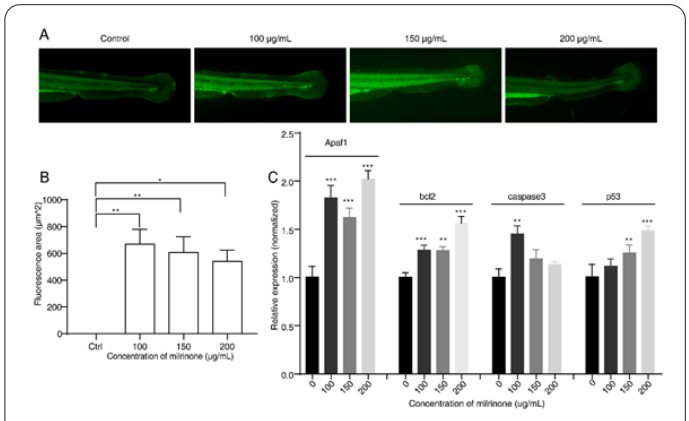

Fig. 5. Milrinone intervention induced apoptosis in zebrafishes. (A) Representative embryo images of 0, 100, 150, and 200 μg/mL milrinone-treated embryos after TUNEL staining. The increasing double-stranded DNA break formation induced fluorescence enhancement in apoptotic cells (indicated by the pyknotic round green fluorescent spots in the images). (B) TUNEL assay quantification of fluorescent signals in zebrafish larvae. Milrinone worsened apoptosis compared to the control. Mean ± SEM; n= 15. *P < 0.05; **P < 0.01. (C) The apoptosis-regulating genes Apaf1, bcl-2 and p53 were up-regulated in embryos intervened by 100, 150, and 200 μg/mL milrinone, respectively. The caspase 3 expression was significantly up-regulated in embryos with 100 μg/mL milrinone treatment. Error bars represent the SD. Student-t test was performed. *P < 0.05; **P < 0.01; ***P<0.001; NS, not significant.

gus monkeys demonstrating that milrinone elevates the cardiotoxicity risk. As expected, there are a number of aberrant expressions of cardiac and vessel development-related transcription factors like GATA4, NKX2.5 and kdrl. Virtually, milrinone-induced embryo cardiotoxicity may be of great value in guiding milrinone applications in pregnancies.

Drug-induced liver injury (DILI) has long been a major clinical concern [22]. Larval zebrafish livers have been shown to have the same function as human livers, which underlies their extensive use for hepatotoxicity assay of xenobiotics. The investigation of milrinone-induced toxicity to zebrafish liver indicated the presence of liver atrophy and down-regulation of *VTG* and *Fabp*10a expression in 150 and 200 μg/mL milrinone-intervened zebrafishes. Moreover, the behavioral retardation of milrinone treated larval was classified as weakness side effect. At the cellular level, TUNEL assay revealed the presence of apoptotic cells in malformed embryos after milrinone intervention. Moreover, the milrinone toxicity causes disturbance in regulation of expression of the apoptosis-associated genes such as *Apaf*1, *p*53 and *bcl*-2.

As far as we are aware, this manuscript presents the embryonic development toxicology of milrinone by ZET. By developing milrinone dosing regimens in zebrafish embryos, we assessed milrinone toxicity through multiple embryonic development indexes like eye area, body length, yolk sac area, HR, pericardial area and liver size, all of which are vital parameters both anatomically and physiologically and are influenced by specified milrinone concentrations. Furthermore, cell apoptosis and gene expression analyses conducted at cellular and molecular levels laid a foundation for the validation of the mechanism underlying milrinone toxicity.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of Fujian Medical University Animal Center.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions
Lingfeng Li, Zhen Chen and Zihe Zheng contributed equally to this work and are co-first authors. Lingfeng Li, Zhen Chen and Zihe Zheng initiated the project and designed the experiment; Xiaofu Dai conducted clinical data collection; Lingfeng Li, Zhen Chen and Zihe Zheng conducted data collation and statistical analysis; Lingfeng Li, Zhen Chen and Zihe Zheng wrote the original manuscript; Xiaofu Dai revised the paper; and all authors reviewed and approved the final manuscript.

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