Cardiotoxicity of Mycotoxin Citrinin and Involvement of MicroRNA-138 in Zebrafish Embryos

Ting-Shuan Wu,* Jiann-Jou Yang,†‡ Feng-Yih Yu,†‡,* and Biing-Hui Liu§,1,2

*Institute of Medicine, †Department of Biomedical Sciences, and ‡Department of Medical Research, Chung Shan Medical University Hospital, Chung Shan Medical University, Taichung, Taiwan; and §Graduate Institute of Toxicology, National Taiwan University, Taipei, Taiwan

1To whom correspondence should be addressed at Graduate Institute of Toxicology, National Taiwan University, No. 1, Sec. 1, Jen-Ai Road, Taipei 10043, Taiwan. E-mail: biingliu@ntu.edu.tw
2These authors contributed equally to this study.

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Citrinin (CTN) is a fungal secondary metabolite that contaminates various foodstuffs and animal feeds; it also exhibits organotoxicity in several animal models. In this study, the zebrafish was used to elucidate the mechanism of CTN cardiotoxicity in developing embryos. Following CTN administration, the gross morphology of the embryonic heart was apparently altered, including heart malformation, pericardial edema, and red blood accumulation. Whole-mount immunostaining and histological analysis of ventricle and atrium indicated incorrect heart looping and reduced size of heart chambers. From the perspective of cardiac function, the heartbeat and blood flow rate of embryos were significantly decreased in the presence of CTN. CTN also modulated the expression of tbx2a and jun B genes, but not that of bmp4 and nkh2.5. Furthermore, the heart areas of CTN-exposed embryos demonstrated an elevated levels of aldh1a2 and cspg2 messenger RNA; these 2 cardiac-related genes are known to be involved in retinoic acid (RA) pathway as well as downstream targets of microRNA-138 (miR-138) in zebrafish. CTN treatment also down-regulated the expression of miR-138. Moreover, overexpression of miR-138 was able to rescue the heart defects generated by CTN. These results support the notion that CTN exposure has a severe impact on heart development, affecting heart morphogenesis through the dysregulation of miR-138, RA signaling, and tbx2a.

Key Words: citrinin; zebrafish embryos; cardiotoxicity; microRNA-138; mycotoxin.

As a secondary metabolite, citrinin (CTN) is generally produced by various fungi, including *Penicillium*, *Monascus*, and *Aspergillus* (Bennett and Klich, 2003). Previous studies have demonstrated that CTN contamination is not only found in cereal and feed but also in *Monascus*-fermented food supplement, which are valued for its ability of cardiovascular protection (Endo, 1979; Su et al., 2003). Several studies have revealed the toxicity of CTN on multiple organs, including kidneys, liver, and intestines (Kitchen et al., 1977; Kumar et al., 2007; Singh et al., 2007a). CTN is also a natural compound showing developmental toxicity, including teratogenicity and embryotoxicity in certain animal models (Reddy et al., 1982). CTN administration caused the malformation of extremities in chick embryos (Ciegler et al., 1977), and the development of rat fetuses was interfered by CTN, resulting in skeletal defects and renal malformation (Singh et al., 2007b).

Our recent study also found that CTN exhibited developmental nephrotoxicity by impairing the glomerulus filtration rate of pronephros in zebrafish (Wu et al., 2012). CTN exposure also reduced the renal blood flow and blood pressure in anesthetized dogs (Krejci et al., 1996). The heart is known to be a major organ responsible for driving blood flow through the circulatory system of animals (Stainier and Fishman, 1994). In addition, the pregnant rat exposed to CTN shows a phenomenon of myocardial fiber degeneration (Singh et al., 2007b). Therefore, we hypothesized that CTN may have unidentified adverse effects on developing hearts, and the cardiotoxicity of CTN as well as its action mechanisms were investigated in the present study.

Zebrafish embryos provide an ideal model system for investigating cardiotoxicity because the uncovered heart of transparent embryos and external fertilization make it easier to observe the embryogenetic process than the fetus in mammals (Hill et al., 2005; Reimers et al., 2006). Besides, the developing zebrafish with a silent heart still survive for at least 5 days after fertilization, but in mammals, impaired heart generally leads to a stillbirth or miscarriage (Dadvand et al., 2009; Kopp et al., 2005). On the other hand, the heart of zebrafish consists of a ventricle and an atrium and these develop rapidly. Heart tube and heartbeat are observed at 24 h postfertilization (hpf), and then tube looping, chamber formation, and blood circulation completed by 72 hpf (Chen et al., 2012; Stainier, 2001).

From a molecular level perspective, certain marker genes, such as tbx2, bmp4, and nkh2.5, playing crucial role in cardiac
mophogenesis are identified in humans as well as in zebrafish (McCulley et al., 2008; Sedletcaia and Evans, 2011; Sultana et al., 2008). The expression of tbx2 gene is negatively regulated by retinoic acid (RA), which profoundly impacts heart formation in mice and zebrafish (Chazaud et al., 1999; Stainier and Fishman, 1992). During RA biosynthesis, retinaldehyde dehydrogenase 2 encoding by aldh1a2 is a key enzyme to transform retinaldehyde into RA (Zhao et al., 1996). When zebrafish is used as a model, aldh1a2 gene is found to be a downstream target of microRNA-138 (miR-138); miR-138 shows the ability in maintaining the stability of atrioventricular (AV) canal and heart tube looping (Morton et al., 2008).

This study demonstrates for the first time that CTN had the ability to impair the structure and function of developing hearts; the playing roles of microRNAs and RA pathway associated with damage were also elucidated by using zebrafish embryos as a model.

MATERIALS AND METHODS

Test species and husbandry. Wild-type (WT) AB strain zebrafish (Danio rerio), heart-specific transgenic line Tg(BMP4:EGFP) (Shentu et al., 2003), and erythrocyte-specific transgenic line Tg(gata1:dsRed) (Traver et al., 2003) were provided by Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS) and raised at 28°C with a 14-h/10-h light/dark cycle. Embryos from the Tg(BMP4:EGFP) and Tg(gata1:dsRed) lines were used for assessing the heart morphology and blood circulation rate, respectively. Healthy and normally developing embryos were selected under a stereomicroscope (Nikon SMZ800) at 6 hpf before subjected to the following experiments.

CTN exposure. Citrinin (Supplementary Figure 1) from Sigma-Aldrich Co (St.Louis, MO) was dissolved in 25% ethanol with 0.01M PBS at a concentration of 10M and stored at −20°C. Depending on the experimental design, the selected zebrafish embryos were arrayed in 96-well plates (1 embryo/well) in 200 μl egg water (60 mg/l ocean salts in distilled water) to appraise the survival rate or put into 24-well plates (10 embryos/l ml egg water/well) for other experiments. Every group was with at least 15 embryos. After maintained at a 28°C incubator, embryos were treated with vehicle (0.125% ethanol in 0.01M PBS) alone or various concentrations of CTN according to the experimental design.

Whole-mount immunostaining. The heart ventricle and atrium were labeled with monoclonal antibodies MF20 and S46, respectively, to determine the chambers’ development (Bader et al., 1982; Stainier and Fishman, 1992). A set of 10 AB line embryos was treated with solvent or CTN (20 and 50 μM) from 24 to 72 hpf, and 3 independent experiments were conducted. Treated embryos were fixed in 0.01M PBS (pH 7.4) containing 4% paraformaldehyde (PFA) for 18h at 4°C. Subsequently, embryos were washed with 0.1% PBST (PBS with 0.1% Tween 20), immersed in 1% blocking solution (Roche, Indianapolis, IN) for 1h at room temperature, and then incubated with monoclonal antibodies MF20 (IgGα) and S46 (IgG) (Developmental Studies Hybridoma Bank, University of Iowa, IA) at 4°C overnight. Following PBST wash, embryos were incubated with either Alex 568 goat anti-mouse IgG2a or Alexa 488 goat anti-mouse IgG (Molecular Probes Inc, Eugene, OR) to develop fluorescent signals. The treated embryos were imaged under Zeiss fluorescence microscope equipped with Rhodamine filter (for MF20 staining) and fluorescein isothiocyanate filter (for S46 staining) (magnification ×200).

Assessment of SV-BA distances in Tg line. CTN-treated embryos of Tg(BMP4:EGFP) were anesthetized by 0.168% Tricaine and mounted on 3% methylcellulose. Photos were taken under Zeiss fluorescence microscope with green fluorescent protein filter (magnification ×200). According to Antkiewicz et al. (2005), the measurement of sinus venosus-bulbus arteriosus (SV-BA) distance and chamber areas were processed with software MetaMorph version 5.0.2r (Universal Imaging Corp, Downingtown, PA).

Histology. For histological transverse sections, WT strain embryos were exposed to solvent or CTN (10 embryos in each treatment) and collected at 72 hpf before fixation in 4% PFA for 18h. The embryos were dehydrated in an ethanol series and then embedded in paraffin for being sectioned into a 5 μm thickness. The resultant sections were sliced and deparaffinized. Subsequently, the slices were dyed for 2min with Mayer’s hemalum solution (Merck), dipped in 0.1% hydrochloride, and then stained with eosin solution (Sigma) for 2min. The slices were sealed with Neo-Mount (Merck) after they were dehydrated to transparency. Finally, the sections were observed and photographed under Zeiss Axioskop microscope (magnification ×200).

Cardiac Functions of Zebrafish Embryos

Heart rate. Zebrafish embryos were treated with solvent, 20 or 50 μM CTN, and the heart rate was assessed at 48 and 72 hpf. A set of 15 embryos was included in each treatment and the heartbeats of each embryo were counted in 20 s under a stereomicroscope. Data obtained from 3 independent experiments were multiplied by 3 to get the rate per minute and then subjected to statistical analysis.

Blood flow rate. For analysis of blood flow, the movement of erythrocyte cells in Tg(gata1:dsRed) was examined at 48 and 72 hpf. The treated embryos were observed on a Zeiss fluorescence microscope with Rhodamine filter and then the erythrocyte number passing through the inner optic circle (Supplementary Figure 2) within 30s was counted. The data were multiplied by 2 to get the blood flow rate per minute. A set of 15 embryos were included in each treated group, and 4 independent experiments were conducted.

Probe construction. To generate the tbx2a (NM_001102384.1) antisense probe, total RNA was extracted from 20 WT embryos at 24 hpf stage and then converted to complementary DNA (cDNAs) with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The following primers (5′-TGTAGCGACACATTTTGGAG-3′ and 5′-ATTTGACACTGCTCGTTTT-3′) were used in PCR to amplify a 801-bp fragment from the cDNA preparations. The PCR product was subcloned into pCRII-TOPO vector (Invitrogen), which was subsequently linearized with BamHI. On the other hand, the placid for generation of bmp4 antisense probe was linearized with XbaI (Walsh and Stainier, 2001). With the linearized plasmids, T7 RNA polymerase plus RNA DIG Labeling mix (Roche) was applied to produce digoxigenin-labeled antisense RNA probes.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was primarily conducted based on the method published by Thissie et al. (1993). CTN-treated embryos from the AB strain (a set of 10 embryos in each group) were fixed in 4% PFA overnight and then removed melanin by using 1% KOH/30% H2O2 (1:1). The embryos were washed with 0.01M PBST and digested with 10 μg/ml proteinase K in PBST. After the embryos were incubated with the antisense probes at 65°C overnight, DIG Nucleic Acid Detection Kit was used to detect the probe (Roche). The embryos were destained with methanol and photographed under Olympus SXX12 stereomicroscope (magnification ×90).

Quantitative PCR for tbx2a, mnx2.5, bmp4, and junB. WT embryos that had been treated with CTN from 24 to 72 hpf were applied to total RNA extraction with Tri-reagent (Invitrogen). For messenger RNA quantification, oligo-dT18 primer and 2 μg of total RNA were included in reverse transcription reaction using Super Script III (Invitrogen), and PCR was followed using designed primers and SYBR Green I Master Mix (Applied Biosystems, Foster city, CA). Elongation factor 1α was used as a reference gene. Data of relative gene expression were obtained from ABI 7000 thermocycler and calculated according to the manufacturer’s description. Each experiment was repeated 3 times using independent batches of embryos and at least 20 embryos were included in each
treated group in every experiment. The primer sets corresponding to each gene were listed in Supplementary Table 1, including tbx2a (NM_001102384.1), ntx2.5 (NM_13142.1), bmp4 (NM_131342.2), and jun B (NM_213556.3).

Partial isolation of embryonic heart. Embryonic hearts were isolated according to Chen et al. (2008) with a slight modification. Sixty embryos of 48 or 72 hpf WT strains, which had been exposed to solvent or CTN since 24 hpf, were collected and anesthetized by 0.4% Tricaine solution. The hearts of zebrafish were separated from their bodies by sheer force generated by sucking and spewing the embryos through a 22-gauge syringe needle. After size fraction of the disrupted embryos under stereomicroscope, the fraction containing hearts was collected by centrifugation at 13 000rpm for 2 min and then subjected to RNA extraction immediately. Four independent experiments were conducted, and a set of 60 embryos were used in each treated group in every experiment.

Real-time PCR for aldh1a2, cspq2, and miR-138. To determine the transcripts of aldh1a2 (NM_131580.1) and cspq2 (XM_002662086.2) levels, partially purified heart fractions as mentioned above were applied to the quantitative PCR. For miR-138 (MIMAT0001868) quantification, specific stem-loop primers designed based on Varkonyi-Gasic et al. (2007) were applied for cDNA production; the quantitative PCR was performed in a reaction containing specific primers, universal probeLibrary probe #21 (Roche), and FastStart Universal probe Master (Roche). U6 small nuclear RNA or miR-26 was used as the reference gene for miRNA analysis. Data of relative gene expression were obtained from ABI 7000 thermocycler and calculated according to the quantitative PCR mentioned above.

Microinjection. Embryos were injected at the 1–2 cell stage with 100 pg of mirVana miR-138 mimics or the negative control No. 1 (nontargeting control, Ambion) into yolk-stream by using Nanoject II injection device (Drummond Scientific, Broomall, PA). The normally developing embryos at 6 hpf were selected under stereomicroscope (Nikon SMZ 800) and subsequently kept till 24 hpf for vehicle and CTN treatment. The cardiac defects of 72 hpf embryos were observed under stereomicroscope. The experiment was independently repeated for 5 times using different batches of embryos and at least 20 embryos were used in each treated group in every experiment.

Statistical analysis. The unpaired 2-tailed Student’s t test was used between 2 groups. One-way ANOVA plus Tukey post hoc test was used between more than 2 groups. Two-way ANOVA was used to analyze the viability. The statistical analyses were performed using GraphPad Prism (version 4.0, GraphPad Software Inc, San Diego, CA). All the data are presented as mean ± SEM. A p value smaller than .05 is considered statistically significant.

RESULTS

Effect of CTN on the Viability of Embryonic Zebrafish

The optimal concentration range and exposure duration were determined by exposing zebrafish embryos at the stage of 24 hpf to various CTN concentrations. The survival rates were also determined at the indicated time points. Although exposure of the embryos to 50 μM CTN displayed a viability of 71.6 ± 10.5% at 72 hpf, none of them were alive at 120 hpf (Fig. 1A). Additionally, CTN at 20 μM lacked an evident lethal effect on embryos by 72 hpf, but the viability reduced to 38.1 ± 8.9% of control at 120 hpf. Above results indicate that the viability of zebrafish embryos decreased when increasing the exposure time and concentrations of CTN.

CTN Caused the Cardiac Defects of Embryos

CTN treatment also induced a dramatic alteration in heart morphology (Fig. 1B). These aberrant changes included incorrect cardiac looping, pericardial edema, and yolk sac edema with blood accumulation. The phenotypic defects of heart chambers were examined closely by performing whole-mount immunostaining with MF20 and S46 antibodies, in which the staining of MF20 identifies the myosin heavy chains in ventricle and S46 recognizes those in the atrium (Stainier and Fishman, 1992). In Figure 2A, although the correct looping and chamber overlapping were observed in solvent-treated embryos (control), the positions of ventricle and atrium in CTN-treated embryos were stretched out and separated. Sizes of the heart chambers also appeared to diminish after 50 μM CTN treatment (Fig. 2A).

The distance between SV and BA is considered an index of cardiac looping (Antkiewicz et al., 2005). Tg(BMP4;EGFP) line, a transgenic fish showing green fluorescence in its heart chambers, was exposed to CTN at 24 hpf, followed by
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Measurement of the SV-BA distance in 72 hpf fish. According to Figure 2B, the SV-BA distance in the control group was 200.2 ± 5.8 μm, yet significantly decreased to 161.8 ± 8.6 μm in 50 μM CTN group. This finding suggests that CTN caused the abnormal heart looping as well as small heart chambers. Further, measuring the areas of ventricular and atrial chambers in Tg(BMP4:EGFP) also demonstrated that CTN exposure reduced the size of developing heart chambers (Supplementary Figure 3).

Phenotypes of hearts were closely examined from histological viewpoints. The heart chambers of zebrafish consist of an outer 1-cell layer of cardiomyocytes, cardiac jelly, and an inner endocardial layer (Stainier, 2001). Nevertheless, treatment of 24 hpf embryos with 50 μM CTN markedly reduced the size of heart chambers and corresponding lumens of either 48 and 72 hpf embryos (Fig. 2C). In addition, 50 μM CTN caused the stacking up of cardiomyocytes by more than 1 layer (inset in Fig. 2C).

CTN Damaged the Cardiovascular Function of Embryonic Zebrafish

An attempt was also made to determine how CTN affects cardiac function by exposing AB strain embryos to vehicle or CTN from 24 hpf and counting the heartbeat at 48 and 72 hpf. The heart rate of control at 72 hpf was 152.1 ± 6.3 beats per minute, and 50 μM CTN administration made the rate significantly decrease to approximately 69.7% of the control (Table 1).

As the heart function is strongly associated with the ability of blood circulation (Duncker and Bache, 2008; Manner 2010), the heart rates after CTN exposure are shown in Table 1. The heart rates were significantly decreased in the 50 μM CTN group compared to the control group. The heart rate at 48 hpf was 131.9 ± 7.8 beats per minute, and at 72 hpf, it was 105.1 ± 2.1 beats per minute.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>48 hpf</th>
<th>72 hpf</th>
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<tbody>
<tr>
<td>Control</td>
<td>144.8 ± 1.2</td>
<td>152.1 ± 6.3</td>
</tr>
<tr>
<td>20 μM CTN</td>
<td>131.9 ± 7.8</td>
<td>137.9 ± 5.4</td>
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<tr>
<td>50 μM CTN</td>
<td>120.6 ± 10.5</td>
<td>105.1 ± 2.1**</td>
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Note. The values of heart rate per minute were presented as the mean ± SEM from 3 independent experiments.

**Significant difference compared with solvent control (p < .01).

Table 1: Heart Rate After CTN Exposure

Fig. 2. Cardiac defects in citrinin (CTN)-treated embryos. The 24 hpf embryos from wild-type (WT) or Tg(BMP4:EGFP) lines were exposed to solvent and 20 and 50 μM of CTN. A, Whole-mount immunostaining of 72 hpf AB line with chamber-specific antibodies MF20 (ventricle, red) and S46 (atrium, green). B, Lateral cardiac images of Tg(BMP4:EGFP) line taken under Zeiss fluorescence microscope (magnification x200). The measured SV-BA distance is indicated by double arrow. The quantitative results are displayed in the lower panel and represented as mean ± SEM from 3 independent experiments. C, Transverse histological sections of cardiac chambers. WT embryos at 24 hpf were exposed to solvent and 50 μM CTN. Transverse sections (5 μm) collected at 48 and 72 hpf were stained with hematoxylin and eosin. The insert figures were enlarged diagrams of partial heart chambers. Magnification x200. Abbreviations: V, ventricle; A, atrium.
et al., 2010), the extent to which CTN adversely affected cardiovascular function was evaluated by using Tg(gata1:dsRed) line with red fluorescence in erythrocytes. Direct visualization of fluorescence intensity in the axial vessels of zebrafish (Belair et al., 2001; Fish et al., 2011). Reduction of fluorescence in CTN-treated 48 hpf embryos suggests that CTN could attenuate the blood flow rate (Fig. 3A). A similar phenomenon of circulation defect was also observed in 72 hpf fish, especially the massive accumulation of red blood cells in pericardium area after 50 μM CTN treatment.

Blood circulation was further closely examined by directly counting the number of erythrocytes passing through the inner optic circle of Tg(gata1:dsRed) in every minute. Although the blood flow rate of 48 hpf control was 206.2 ± 9.6 erythrocytes/min, the rates in 20 and 50 μM CTN-treated embryos were significantly reduced to 76.6% and 59.2% of the control group, respectively (Fig. 3B). The systemic circulation of red blood cells was nearly undetected in 72 hpf fish exposed to 50 μM CTN. Above results indicate that CTN modifies the cardiac function of embryonic fish in terms of heartbeat rate and blood circulation.

**CTN Altered the Expression of Heart-Related Genes**

Widely considered as heart-related genes, tbx2a, nkkx2.5, bmp4, and junB are strongly associated with the development of the heart chamber, valve, and sarcomeric Z-disc in zebrafish (McCulley et al., 2008; Sultana et al., 2008). CTN downregulated the level of tbx2a transcript to 30.8 ± 0.1% of the control group and upregulated junB expression to 10.9 ± 0.5-folds of 72 hpf control (Fig. 4); both messenger RNA (mRNA) signals of bmp4 and nkkx2.5 genes remained unchanged after 50 μM CTN administration.

On the other hand, the incorrect heart looping induced by CTN was restored after coexposure of CTN with metoprolol (MET), a clinical medicine widely used in several human cardiovascular diseases (Fig. 5A). Simultaneously, the presence of MET partially recovered the tbx2a and junB expression altered by 50 μM CTN.
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CTN (Fig. 5B), indicating that both tbx2a and junB genes are involved in CTN-induced heart malformation. MET also prevented the mortality of embryos induced by CTN; conversely, isoproterenol and enalapril maleate, 2 other clinical medicines, did not exhibit the similar effect (Supplementary Figure 4). Both bmp4 and tbx2a genes are essential in regulating the formation of heart AV canal, which functions as an organizing tissue for chamber alignment and septation (Patra et al., 2011; Verhoeven et al., 2011). According to Figure 6, signals of bmp4 and tbx2a were both detected and restricted in the AV canal of the control heart by applying whole-mount in situ hybridization. Similarly, in the presence of 50 μM CTN, the bmp4 transcript was still located in the AV canal despite the abnormal looping and smaller chamber lumens. However, the tbx2a signal was completely undetected in the region of AV canal.

CTN Modulated the RA Signaling Pathway in Control of Cardiac Patterning

Because CTN treatment modified the level and distribution of tbx2a transcripts in embryonic hearts and tbx2 gene with RA response element in its promoter region is negatively regulated by RA (Sakabe et al., 2012; Sasagawa et al., 2002), the possible role of RA signaling in CTN-triggered cardiac defects was further studied herein. During RA biosynthesis, retinaldehyde dehydrogenase, the product of aldh1a2 gene, is responsible for the conversion of retinaldehyde into RA; on the other hand, cspg2 gene encoding versican is a downstream target of RA (Morton et al., 2008; Zhao et al., 1996). Following exposure of zebrafish embryos to 50 μM CTN from 24 to 72 hpf, the mRNA levels in embryonic hearts revealed that aldh1a2 and cspg2 expressions were significantly upregulated to 2.3- and 2.4-folds of control, respectively (Fig. 7A). Morton et al. (2008) has mentioned that in the zebrafish model, both aldh1a2 and cspg2 are target genes of miR-138, and miR-138 modulates cardiac patterning during embryonic development. After exposed to 50 μM CTN, the levels of miR-138 transcripts in 48 and 72 hpf embryos were significantly decreased to 38.8±6.1% and 35.5±8.2% of control, respectively (Fig. 7B).

We also examined the levels of miR-129-3p and miR-218a, 2 important microRNAs involved in the looping and formation of the heart tube (Cao et al., 2012; Fish et al., 2011). Although CTN did not modify the miR-129-3p level, it drove the repression of miR-218a (Supplementary Figure 5). Nevertheless, further investigation revealed that CTN had no effect on the miR-218a target genes, robo1 and robo2 (Supplementary Figure 6). These data imply that the miR-138/RA/tbx2a pathway, but not miR-218a/Slit-Robo signaling, may be crucial in CTN-induced cardiac defects.

CTN-Induced Heart Defects Could Be Reversed by miR-138 Mimics

Because miR-138 targets the 3′UTR of aldh1a2 and cspg2 mRNA (Morton et al., 2008) and miR-138 expression was decreased in the CTN-treated heart (Fig. 7B), we further
addressed whether a loss of miR-138 was indeed a causative factor for the observed cardiac defect induced by CTN. WT embryos were injected with the synthetic mimics for miR-138 or negative control (NC) before exposed to 30 or 50 μM of CTN, and the representative heart phenotypes from different treatment were demonstrated in Figure 8A. For those embryos injected with NC mimic, CTN at concentrations of 30 and 50 μM led to 72.5 ± 7.2% and 99.2 ± 0.8% of embryos showing heart defects, including abnormal heart looping, pericardial edema, and cardiac ischemia (Fig. 8B). After injected with the miR-138 mimic, the percentages of defective embryos exposed to 30 and 50 μM CTN were significantly reduced to 15.0 ± 7.3% and 54.3 ± 14.5%, respectively.

FIG. 7. Citrinin (CTN) modulated the expression of miR-138 and its target genes in embryonic heart. Wild-type embryos at 24 hpf were treated with solvent or 50 μM CTN, and then heart samples were collected for RNA extraction and real-time PCR according to Materials and Methods section. A, The levels of aldh1a2 and cspg2 transcripts were determined at 72 hpf. B, The expression of miR-138 was determined at 48 and 72 hpf. Each set of data represents mean ± SEM from 3 independent experiments. *p < 0.05 and **p < 0.01, significantly different between compared groups.

DISCUSSION

Mycotoxin CTN is a known contaminant in a variety of cereals and Monascus fermentation products. The latter generally contains substantially higher levels of CTN, because in Asian countries, Monascus species is intentionally inoculated to generate dietary products with a special color, flavor, and even cholesterol-reducing compound monacolin K (generic name: lovastatin) (Endo, 1979; Su et al., 2003). These fermented products are also popularly used as alternative medicines in Western countries (Heber et al., 1999; Kumari et al., 2009). Monascus samples collected in the Netherlands were all contaminated with CTN at concentrations ranging from 0.2 to 17.1 ppm (Sabater-Vilar et al., 1999). A Taiwan government survey indicated that at least 15% of the examined samples had CTN levels exceeding 10.0 ppm (Liao et al., 2010). Therefore, in this study, up to 50 μM (12.5 ppm) of CTN was used to reflect how long-term consumption of CTN-contaminated food affects the early heart development.

CTN altered the phenotypes of developing heart, including abnormal tube looping, reduced chamber sizes, and pericardial edema (Figs. 1 and 2). Erythrocytes were also found to...
accumulate in the pericardial cavity of 72 hpf Tg line after CTN exposure; moreover, the blood flow rate in inner optic circle was significantly reduced (Fig. 3). These data imply a phenomenon similar to cardiac ischemia. In mouse and porcine models, myocardium subjected to ischemia and reperfusion showed a significant increase in \(\text{junB}\) levels and activity (Alfonso-Jaume et al., 2006; Brand et al., 1992). We also found that \(\text{junB}\) expression was dramatically elevated after CTN treatment (Fig. 4). The role of \(\text{junB}\) in CTN-induced myocardial infarction is unclear; increasing JunB and associated AP-1 activities may transcriptionally stimulate certain target genes contributing to the protection of ventricular dysfunction and consequently repair processes (Alfonso-Jaume et al., 2006; Brand et al., 1992).

The presence of heart medicine MET, a \(\beta\)-adrenergic receptor antagonist, restored \(\text{tbx2a}\) and \(\text{JunB}\) mRNA levels, recovered the morphology, and reduced the mortality of embryos treated with CTN (Fig. 5 and Supplementary Figure 4). These data further confirm the involvement of \(\text{tbx2a}\) and \(\text{JunB}\) gene in CTN-induced toxicity. Nevertheless, whether the action mechanism of MET on blocking CTN toxicity is correlated with \(\beta\)-adrenergic receptors required more evidence. \(\beta\)-Blockers, such as MET, are able to improve clinical outcome when administered early after acute myocardial infarction (Hjalmarson et al., 1981; Ibanez et al., 2007). Additionally, there are dramatical success in treating mild-to-moderate chronic heart failure with \(\beta\)-adrenergic blocking agents (Bristow, 2000). MET is also reported to rescue the heart failure in zebrafish embryos exposed to phytotoxin aristolochic acid, even though the molecular function of MET is not illustrated (Huang et al., 2007).

CTN markedly decreased \(\text{tbx2a}\) transcript levels in embryos (Fig. 4). Similarly, the immunostaining signal of \(\text{tbx2a}\) was undetected in the AV canal of the heart (Fig. 6). The product of \(\text{tbx2}\) gene is essential in AV canal development and heart valve formation in mice and zebrafish (Harrelson et al., 2004; Sedletcaia and Evans, 2011; Singh et al., 2012). It has been well known that the expression of \(\text{tbx2a}\) gene is positively regulated by \(\text{bmp4}\); the BMP family is reported to direct expression of \(\text{tbx2}\) mRNA, but not those of \(\text{bmp4}\) (Figs. 4 and 6), suggesting that CTN modulates the \(\text{tbx2a}\) expression without interfering the upstream \(\text{bmp4}\). This finding is consistent with the observation of Ribeiro et al. (2007) in which decreasing \(\text{tbx2a}\) expression in zebrafish leads to incorrect looping and chamber formation; however, the \(\text{bmp4}\) levels remain normal in the embryonic heart.

The \(\text{tbx2}\) gene expression is also known to be negatively regulated by RA, which binds to the RA response element in the \(\text{tbx2}\) promoter region (Sakabe et al., 2012; Sasagawa et al., 2002). CTN exposure upregulated the activity of \(\text{aldh1a2}\) and \(\text{cspg2}\), 2 important genes involved in the RA signaling pathway, in embryonic hearts (Fig. 7A). It suggests that CTN might elevate the RA level, which subsequently contributed to \(\text{tbx2}\) downregulation. The retinaldehyde dehydrogenase 2 encoded by \(\text{aldh1a2}\) is located in developing heart valves and AV canal in both zebrafish and mouse (Morton et al., 2008; Niederreither et al., 2002); it is also required in visual development and neuronal differentiation in zebrafish (Le et al., 2012; Linville et al., 2004). On the other hand, Mjaatvedt et al. (1998) mentioned that normal expression of \(\text{cspg2}\) gene is required for the successful development of cardiac chamber and endocardial cushion swellings. Thus, in addition to \(\text{tbx2}, \text{cspg2}\) activated by CTN may be another direct factor contributing to cardiac defects.

While promoting \(\text{aldh1a2}\) and \(\text{cspg2}\) mRNA levels, CTN also suppressed the miR-138 expression in heart samples (Fig. 7B). It supports the finding of Morton et al. (2008) that miR-138 modulates \(\text{aldh1a2}\) and \(\text{cspg2}\) gene activities during cardiac patterning of zebrafish. Moreover, the injection of synthetic miR-138 mimic into embryos rescued the CTN-induced cardiac malformation (Fig. 8), indicating the adverse influence of CTN on developing hearts partly due to interfere with miR-138 balance. However, it remains unknown how CTN interacts with miR-138 in the embryonic heart because few reports reveal the upstream regulation of miR-138 in any species. In addition to heart patterning in fish, the miR-138 in rodents
plays roles in dendritic spine morphogenesis and recognition memory (Siegel et al., 2009; Tatro et al., 2013). This microRNA is also recognized as a potential tumor suppressor gene in several cancer cell models (Jin et al., 2013; Wang et al., 2012). Therefore, whether miR-138 attenuated by CTN has other physiological/pathological effects is an interesting issue for the future study.

As is well known, human cardiovascular disease is strongly correlated with kidney dysfunction (Amann et al., 2006; Couser and Riella, 2011). Our previous study also shows that CTN impairs the kidney function in embryonic zebrafish (Wu et al., 2012). When embryos were treated with 50 μM CTN, the 72 hpf heart displayed phenotypes of abnormal looping and small lumens of ventricle and atrium (Figs. 1B and 2). However, CTN at 50 μM did not modify the gross morphology of fish kidneys (Wu et al., 2012). Furthermore, in the heart area, the tbx2a mRNA signal was significantly decreased after 50 μM CTN exposure (Figs. 4 and 6); conversely, the same amount of CTN did not affect the expression level of specific renal genes in glomeruli or pronephric tubules (Wu et al., 2012). Comparing the results from both organs revealed that CTN exerts stronger adverse effects on developing heart through suppressing miR-138 expression and subsequently promotes RA-related aldh1a2 and cspg2 activities and attenuates tbx2a levels; all of these factors are well known to be strongly associated with cardiac morphogenesis in rodents and fish. Understanding the toxicological mechanism driven by CTN in vivo could provide a solid base to evaluate the detrimental effects of mycotoxin on the general public, including pregnant women and developing individuals.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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