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The toxic effect of Amiodarone on valve formation in the developing heart of zebrafish embryos

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ABSTRACT

Background: Amiodarone is a class D drug given to treat arrhythmia, including pregnant women, but its effects on the developing heart have not been studied. Although some studies have suggested that this drug is safe for fetuses, they have been conducted on mothers with fetuses at or beyond six months of gestational age.
Results: The occurrence of valve defect was positively proportional to Amiodarone concentrations over 9 μM, but not lower than 6 μM. Ectopic overexpression of *versican* was observed at the atrioventricular canal of the Amiodarone-treated embryos at 15 μM (EC_{50}). *VE-cadherin* (*cdh5*), normally downregulated at the endocardial cushion, was also ectopically overexpressed in the Amiodarone-treated embryos. Knockdown of either *versican* or *cdh5* in the Amiodarone-treated embryos could rescue the valve defect caused by Amiodarone.
Conclusions: By inducing *versican* ectopical overexpression, leading, in turn, to *cdh5* ectopical overexpression, Amiodarone treatment causes failure of cardiac valve formation in zebrafish embryos.

1. Introduction

Amiodarone is categorized as a type III antiarrhythmic drug [1,2]. It is considered a broad-spectrum antiarrhythmic agent [3] because it has multiple and complex effects on the electrical activity of the heart. As such, Amiodarone is effective in treating tachyarrhymias, including re-entry supraventricular tachycardias, ventricular tachycardia, atrial arrhythmias and ventricular fibrillation [4]. While Amiodarone is considered the antiarrhythmic treatment of choice, it is classified as a category D drug. Consequently, caution should be exercised before using Amiodarone for pregnant women, as it causes embryonic hypothyroidism and hyperthyroidism [5,6]. In contrast, Valensise et al. [7] showed that long-term use of Amiodarone has no effect on embryogenesis. In a clinical study [8], no developmental effects, except one case of hypothyroidism, was demonstrated. However, these studies focused on embryos older than 6 months when most organs, including the heart, have been completely formed. There is no epidemiological data for women who were taking Amiodarone when they inadvertently became pregnant. Still, based on the evidence at hand, it is important to know whether Amiodarone could be toxic for embryos at an early developmental stage because the half-life of Amiodarone is reported to be 26–107 days [9], and Amiodarone could be prescribed in the case of undetected pregnancy or pregnancy within the gestational period.

To study the toxicity of Amiodarone on heart development, we used zebrafish as a system model since the transparency of embryos allows us to directly observe cardiac development without invasive procedures. As such, zebrafish is an excellent organism to study cardiovascular genetics and defects [10]. Two zebrafish heart-specific fluorescence transgenic lines are available for the *in vivo* study of cardiac development: *Tg(cmlc2:EGFP)* with heart-specific green fluorescence [11] and *Tg(cmlc2:HcRFP)* with heart-specific infrared

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emission [12,13]. In addition, early-stage cardiac development of zebrafish is similar to that of human in many respects, such as migration of cardiac precursor cells towards the central line, heart tube formation, early chamber formation and the looping process.

In our studies, we found that Amiodarone caused defects in cardiac valve formation of zebrafish embryos. We also revealed that the molecular mechanism underlying such defects involves overexpression of both *versican* and *VE-cadherin5* (*cdh5*) at the atrioventricular canal (AVC). Therefore, we concluded, that ectopic expression of *versican* and *cdh5* induced by Amiodarone caused defects impeding normal heart valve development in zebrafish.

2. Materials and methods

2.1. Observation of zebrafish transgenic lines and heart development

The zebrafish AB strain, as well as transgenic lines *Tg*(*cmlc2:HcRFP*) and *Tg*(*cmlc2:EGFP*), were cultured as previously described. Heart formation was observed under fluorescent stereomicroscopy (MZ FLIII, Leica). Valve formation was observed *in vivo* by using Harmonic Generation Microscopy assisted by Two-Photon Fluorescence Microscopy (HGM/2PF) [13]. The excitation source was a femtosecond Cr:forsterite laser with an output wavelength of 1230 nm. Second harmonic generation (SHG) and third harmonic generation (THG) [12,14] were applied to observe valve development in transgenic zebrafish *Tg(cmlc2:HcRFP) in vivo*. RFP marked the myocardial cells, while THG (410 nm) marked all cells in yellow, and SHG (615 nm) marked the skeletal and cardiac muscles in green. Paraffin sectioning with Hematoxylin and Eosin (H&E) staining was also used to perform histochemical analysis of the heart.

2.2. Drug treatment with zebrafish embryos

Amiodarone (Sigma) was dissolved in water at 65 °C for 2 h and stocked as 900 μM at 4 °C. Before use, the solution was re-dissolved at 65 °C for 1 h. In the control group, 100 embryos were placed in a 9 cm dish filled with a volume of 30 ml embryo medium containing 0.2 mM 1-phenyl-2-thio-urea (Sigma). In the experimental group, the protocol was identical to the control group, except that embryos at different stages were treated with concentrations of Amiodarone that ranged from 3 to 30 μM, and embryos were exposed to treatment from 12 to 84 h (Supplementary Fig. 1). Long-term treatment during 12–72 hpf included the specification stage of valve formation at 36–55 hpf and the invagination stage of valve formation at 55 hpf. Treatment during 12–48 hpf was used to examine the gene markers *versican* and *cdh5* which expressed at the AVC. During treatment, Amiodarone was refreshed every 24 h, and after treatment, embryos were washed twice with embryo medium, collected into a new 9 cm dish, and then incubated at 28 °C.

Ion channel inhibition was achieved by treating embryos with 3.5 mM 4-Amiopyridine (potassium channel blocker; Sigma), 200 mg/L Nifedipine (calcium channel blocker; Sigma), 15 mg/L Lidocaine (sodium channel blocker; Sigma) or 20 mg/L Propranolol (beta-adrenergic receptor blocker; Sigma), and then fixing the embryos by 4% paraformaldehyde. 100 embryos were placed in a 9 cm dish filled with a volume of 30 ml embryo medium containing these inhibitors from 12 to 48 hpf.

2.3. Knockdown experiments

The following morpholino nucleic acid oligomers (MOs) were purchased from GeneTools (USA): *versican*-MO (CTGAAACACCCATGGGAGTGGACAT); *cdh5*-MO (TTTACAAGACCGTCTCCTTTCCAA) [15,16]; and standard control-MO (CCTCTTACCTCAGTTACAATTTATA). All MOs were prepared at a stock concentration of 1 mM and diluted to the desired concentration, specifically, 8, 12 and 16 ng for *versican*-MO; 4, 8, 12 and 16 ng for *control*-MO; and 0.8, 1.2, 1.6 and 2 ng for *cdh5*-MO. The standard control-MO served as negative control (Supplementary Figs. 4A vs. B).

2.4. Whole-mount in situ hybridization (WISH)

WISH was performed as previously described [17]. Riboprobe of *cdh5* was prepared by cloning its partial DNA fragment, while riboprobe of *versican* was provided by Haramis [18].

2.5. Western blot analysis

The embryos were dechorionated and deyolked with two extra washing steps as described in Link et al. [19]. Deyolked samples were dissolved in 2 μl of 2× SDS sample buffer for each embryo and incubated for 5 min at 95 °C. After full-speed centrifugation for 1 min in a microcentrifuge to remove insoluble particles, total proteins extracted from embryos were analyzed on a 12% SDS-PAGE gel, and Western blot analysis was performed [20] using antiserum against mouse Cdh5 (15; 1:10,000). Anti-α-tubulin and anti-β-actin served as a protein loading control.

Table 1
The percentages of defective phenotypes of zebrafish embryos treated with different concentrations of Amiodarone for various exposure times.

Concentration	Treatment duration	The percentages of phenotypes	
		Blood regurgitation	Pericardiac edema
0 μM	–	2/200 (1%)	7/200 (3.5%)
3 μM	(12 hpf–48 hpf)	4/320 (1.3%)	5/313 (1.6%)
6 μM	(12 hpf–48 hpf)	20/416 (4.6%)	15/384 (3.9%)
9 μM	(12 hpf–48 hpf)	168/398 (42.2%)	72/398 (18.1%)
12 μM	(12 hpf–48 hpf)	177/331 (53.5%)	53/304 (17.4%)
15 μM	(12 hpf–48 hpf)	351/638 (55.0%)	166/638 (26.0%)
30 μM	(12 hpf–48 hpf)	188/295 (63.7%)	78/237 (32.9%)
3 μM	(12 hpf–72 hpf)	3/317 (0.9%)	11/317 (3.5%)
6 μM	(12 hpf–72 hpf)	27/403 (6.7%)	43/372 (11.6%)
9 μM	(12 hpf–72 hpf)	189/394 (47.9%)	174/368 (47.3%)
12 μM	(12 hpf–72 hpf)	103/216 (47.7%)	116/237 (48.9%)
15 μM	(12 hpf–72 hpf)	78/156 (50%)	79/156 (50.6%)
30 μM	(12 hpf–72 hpf)	–[a]	–

The medium and drugs were renewal every 12 h.
[a] All embryos were lethal.

2.6. Statistics

All values for statistical significance represent the mean ± standard deviation (S.D.). We arrived at means by *t*-test with significant difference of $P < 0.05$. For dose response curve, the formula we used is $Y = (\text{Top} - \text{Bottom})/(1+10\log EC_{50} - X)$Hill slope. The variable Hill slope describes the steepness of the curve. A standard dose response curve has a hill slope of ±1. Means and standard errors were determined according to at least three independent experimental replicates. Regression curves are generated using the MasterPlex non-linear regression analysis for 4 parameter logistic models software.

3. Results

3.1. The toxicity and lethal dosage of zebrafish embryos treated with Amiodarone

First, we studied the half-lethal concentration (LC_{50}) of Amiodarone treatment at different concentrations and under different exposure times. The percentage of embryos suffering acute toxicity from lethal dosage at each treatment concentration was calculated. As shown in Supplementary Fig. 2, the rate of lethality resulting from Amiodarone dosages from 9 μM to 18 μM for an exposure of 36 h was no more than 10%. In contrast, the rates of lethality resulting from Amiodarone dosages at 15 μM and 18 μM for an exposure of 60 h were 36.5 and 38%, respectively. When embryos were treated with 30 μM Amiodarone, the rate of lethality exceeded 80%, making it impossible to observe heart development during later stages. Thus, when higher concentrations of Amiodarone are coupled with longer exposure times, the rates of lethality among treated embryos increased substantially. Additionally, we also calculated the occurrence percentages of such heart defects as slow heartbeat, blood regurgitation and pericardiac edema in zebrafish embryos treated with different concentrations of Amiodarone at different exposure times. As shown in Table 1, compared to control group, no increase in heart defects was observed in embryos treated with lower concentrations of Amiodarone, such as 3 μM and 6 μM for 36 and 60 h. However, the occurrence percentages of pericardiac edema and blood regurgitation were dramatically increased if embryos were treated with higher concentrations of Amiodarone, such as 9 μM, 12 μM and 15 μM for 36 and 60 h. We determined that the half maximal effective concentration (EC_{50}) of Amiodarone is 15 μM and that the exposure time is either 36 h (from 12–48 hpf) or 60 h (from 12–60 hpf). We chose a 15 μM concentration of Amiodarone for further experiments in this study for the following reasons: (I) Zebrafish heart development is completed at 72 hpf. (II) The LC_{50} of Amiodarone for an incubation of 60 h ranged from 18 to 24 μM. (III) The EC_{50} of Amiodarone is

15 μM, which is below the LC_{50} (Supplementary Fig. 3). (IV) This concentration is close to the EC_{50} which avoids defects that are caused by nonspecific toxicity, and it is also easy for us to quantify.

3.2. Amiodarone caused abnormal heartbeat and defective valve formation in zebrafish embryos

Embryos were treated with 15 μM Amiodarone to observe its effect on zebrafish development. In contrast with control embryos, embryos treated with 15 μM Amiodarone from 12 to 48 hpf had shorter axes, smaller heads, and more delayed development (Fig. 1A vs. D). No obvious defects in the pericardiac cavity (Fig. 1B vs. E) were observed, but 82.3% of the embryos showed tail shrinkage (Fig. 1C vs. F). If embryos were continuously treated with 15 μM Amiodarone from 12 to 72 hpf, the embryos showed phenotypes similar to those treated at 48 hpf. However, these embryos were smaller (Fig. 1G vs. J) and showed pericardiac edema (Fig. 1H vs. K), some tail shrinkage (Fig. 1I vs. L) and some ulceration to the venous plexus (Fig. 1L). We noted that pericardiac edema and tail shrinkage were both dependent on the concentration of Amiodarone

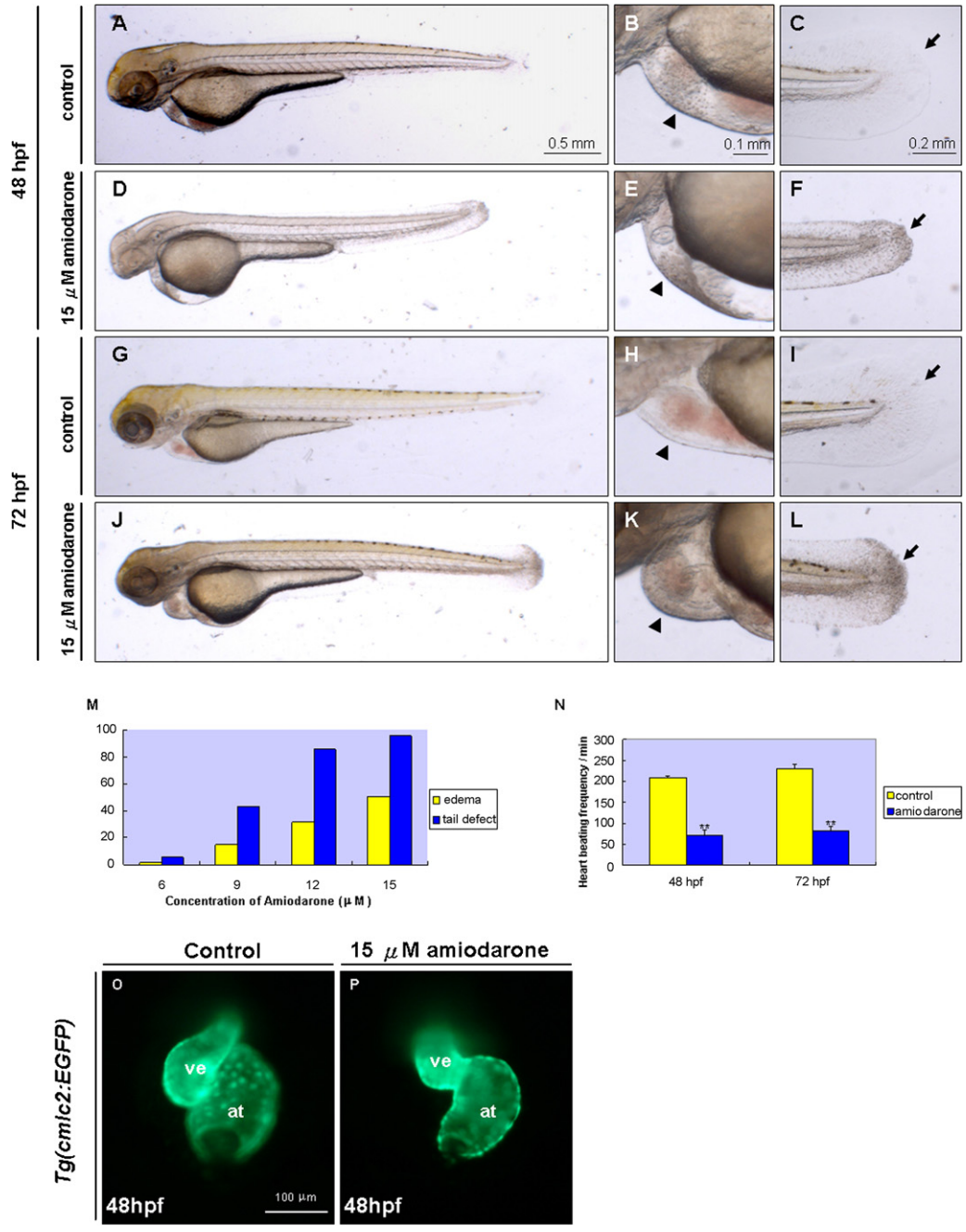

Fig. 1. Defective phenotypes of embryos caused by Amiodarone treatment. Zebrafish embryos were treated with 15 μM Amiodarone starting at 12 hpf, and the phenotypes of the control (A–C and G–I) and treated (D–F and J–L) groups were compared. Compared to the control group, embryos treated with Amiodarone during 12–48 hpf displayed defective phenotypes, such as shorter axes, smaller heads, developmental delay (A vs. D), and cell necrosis at the pericardial membrane (B vs. E, triangle) and tail-fin (C vs. F, arrow). Embryos treated with Amiodarone from 12–72 hpf displayed phenotypes (G vs. J; H vs. K; and I vs. L) similar to the embryos treated at 12–48 hpf, except with higher ratio of phenotype occurrence and a swollen pericardial cavity (K). The occurrence of defective phenotypes of embryos treated with Amiodarone from 12–72 hpf was calculated (L). The rate of heartbeat (in bpm) was counted in the embryos treated with 15 μM Amiodarone for 36 and 60 h (M). Data are presented as mean ± S.D. Embryos derived from transgenic line *Tg(cmlc2:EGFP)* were observed under fluorescent microscopy, and the incomplete looping of developing heart could be observed in embryos treated with 15 μM Amiodarone during 48 hpf (O vs. P).** indicates the significant difference at the level of $P < 0.05$. ve, ventricle; at, atrium.

(Fig. 1M). We found that the heart rate was also greatly reduced in the embryos treated continuously with 15 μM Amiodarone for 36 and 60 h (Fig. 1N). In embryos treated with Amiodarone for 36 h, there was evidence of arterial/venous occlusion and ventricular arrhythmia. In a morphological examination of the heart, we also found that looping of the developing heart was incomplete in embryos treated with 15 μM Amiodarone during 48 hpf (Fig. 1O vs. P). Specifically, the endocardial cushion, a subset of cells found in the developing heart tube that gives rise to the heart's valves and septa, begins to function at 72 hpf, which is when we noticed blood regurgitation between atrium and ventricle in the Amiodarone-treated embryos (see attached movie 1 vs. movie 2).

Endocardial cushion cells proliferate at the AV canal, and the process of specification begins during 36 to 55 hpf. Therefore, we treated embryos with 15 μM Amiodarone during 22–34, 34–46 and 46–58 hpf and then examined for blood regurgitation both at 48 and 72 hpf. When we examined embryos at 48 hpf, we found that the occurrence of blood regurgitation was dramatically increased in the embryos treated with Amiodarone during 34–46 hpf and during 46–58 hpf, compared to control embryos (Supplementary Table 1). Such results suggested that Amiodarone causes blood regurgitation of zebrafish embryos during, but not prior to, valve formation. Importantly, when Amiodarone treatment was stopped either at 36 or 58 hpf, most defective embryos displaying the blood regurgitation phenotype could be rescued by 72 hpf (Supplementary Table 1), indicating that heart defect induced by Amiodarone can also be abolished in the absence of Amiodarone.

Since the most important function of valves is the blockage of blood regurgitation from ventricle to atrium, we further examined whether a defect of valve development occurred in the process of heart development. Histochemical staining on the paraffin sectioning of 72-hpf zebrafish hearts revealed that the AVC endocardial cushion-forming region (ECFR) showed a bulged structure in the untreated embryos (Fig. 2B). These endocardial cushion cells originated from endocardial cells migrating from the AVC towards the extracellular matrix (ECM), where these cells started to aggregate and proliferate. However, in the Amiodarone-treated embryos, the ECRF failed to form at the corresponding position of AVC, and the endocardial cells remained a structure formed by a single layer of cells (Fig. 2D). These results indicated that Amiodarone treatment caused abnormal valve development in zebrafish embryo hearts.

3.3. Cardiac valves of zebrafish were directly observed in vivo

By using HGH/2PF to examine the zebrafish embryos derived from transgenic line *Tg(cmlc2:HcRFP)*, we could easily observe the dynamics of valve development *in vivo*. For example, at 48 hpf, there was a single layer of cells in the endocardium at the AVC (Fig. 3A). At 72 hpf, a bulged structure was observed at the AVC (Fig. 3B), and the endocardial cells continued to move towards cardiac jelly and gradually elongated to form the structure of valves at 96 hpf. Finally, at 87 hpf, the elongated structure was protruding towards the ventricle (Fig. 3C). However, if we treated these embryos during 12–87 hpf with 300 μM CsA, a drug known to repress epithelial-mesenchymal transition (EMT) and thus cause valve defect [21], we found that valves were not formed, which, again, could be clearly observed at 87 hpf under HGH/2PF (Fig. 3H). Interestingly, when we treated zebrafish embryos with 15 μM Amiodarone during 12–48 hpf, no difference between treated and untreated embryos was noted in endocardial cells at the AVC where only a single layer of cells was observed (Fig. 3A vs. E). However, when embryos were treated with the same dosage of Amiodarone during 12–72 hpf, only a small aggregation of cells could be seen at the upper valve site and no valve structure at the lower site (Fig. 3B). Furthermore, compared to untreated embryos (Fig. 3C), embryos treated with Amiodarone during 12–87 hpf displayed almost no cellular aggregation at the valve region (Fig. 3G).

3.4. Loss of endocardial cushion cells resulted from Amiodarone treatment, not apoptosis

Long-term treatment of embryos with Amiodarone resulted in defective valves (Fig. 2D). This defect might have been caused by Amiodarone-induced apoptosis, which, in turn, would reduce the number of caudal fin and endocardial cushion cells. Therefore, to confirm Amiodarone-induced apoptosis in the endocardial cushion areas, the TUNEL assay was applied. In the 72-hpf control group without Amiodarone treatment, the cardiac and marginal regions were transparent without any apoptotic signals (Supplementary Figs. 4A and B). Treatment with 15 μM Amiodarone from 12 hpf showed some signals of apoptosis in the marginal region of the trunk (Supplementary Fig. 4D), but stronger signals in the tail. At the same time, however, no apoptotic signals were observed in the cardiac area (Supplementary Fig. 4C). Similarly, there were no apoptotic signals in the 87-hpf control group (Supplementary Figs. 4E and F). At 87 hpf, the embryos that had been treated with 15 μM Amiodarone from 12 hpf showed severe apoptosis in the tail region, whereas apoptotic signals were still undetectable in the cardiac region (Supplementary Fig. 4G). Thus, the valve defect observed in embryos undergoing long-term Amiodarone treatment did not result from Amiodarone-induced apoptosis, which, in turn, would have reduced the number of caudal fin and endocardial cushion cells. Instead, it appears that Amiodarone acts directly to cause the loss of endocardial cushion cells.

3.5. Amiodarone treatment caused ectopic overexpression of versican

Versican, a gene involved in cell migration, is expressed in the myocardium [22,23] and endocardium [18], respectively, at AVC in zebrafish at 48 hpf. Therefore, we performed WISH to observe the expression of *versican*, which is indicative of valve formation, in embryos treated with Amiodarone. In wild-type embryos, *versican* was normally expressed at AVC at 48 hpf (Fig. 4A). However, embryos treated with 15 μM Amiodarone during 12–48 hpf showed massive ectopic overexpression of *versican* in ventricle, atrium, and at AVC (Fig. 4B). Similarly, *versican* was expressed restrictedly at AVC at 72 hpf in the untreated embryos (Fig. 4C). However, in the embryos treated with 15 μM Amiodarone during 12–72 hpf, *versican* was again found to be expressed ectopically in ventricle, atrium, and at AVC (Fig. 4D). We note that the ectopic overexpression of *versican* at AVC was observed irrespective of the embryonic stage during which Amiodarone treatment was begun (12–7 2hpf, 36–72 hpf, 36–48 hpf, 48–60 hpf, or 60–72 hpf) (Fig. 4F–J). In fact, *versican* was overexpressed ectopically at AVC, even in embryos treated with Amiodarone at 84–96 hpf, after valves had been formed (Fig. 4K vs. L). This line of evidence indicated that Amiodarone treatment enables embryonic overexpression of the genes involved in endocardial cell migration in zebrafish heart, which, in turn, suggests that Amiodarone may impede the normal development of heart valve formation.

3.6. Amiodarone treatment at specification and invagination stages caused versican to overexpress at AVC and repressed valve development

Guided by the hypothesis that Amiodarone impedes proper heart valve formation by causing the overexpression of *versican*, we further investigated the critical stage(s) at which Amiodarone affects heart valve development. To accomplish this, we treated embryos with Amiodarone at different developmental stages and examined the embryos for *versican* expression at AVC with WISH

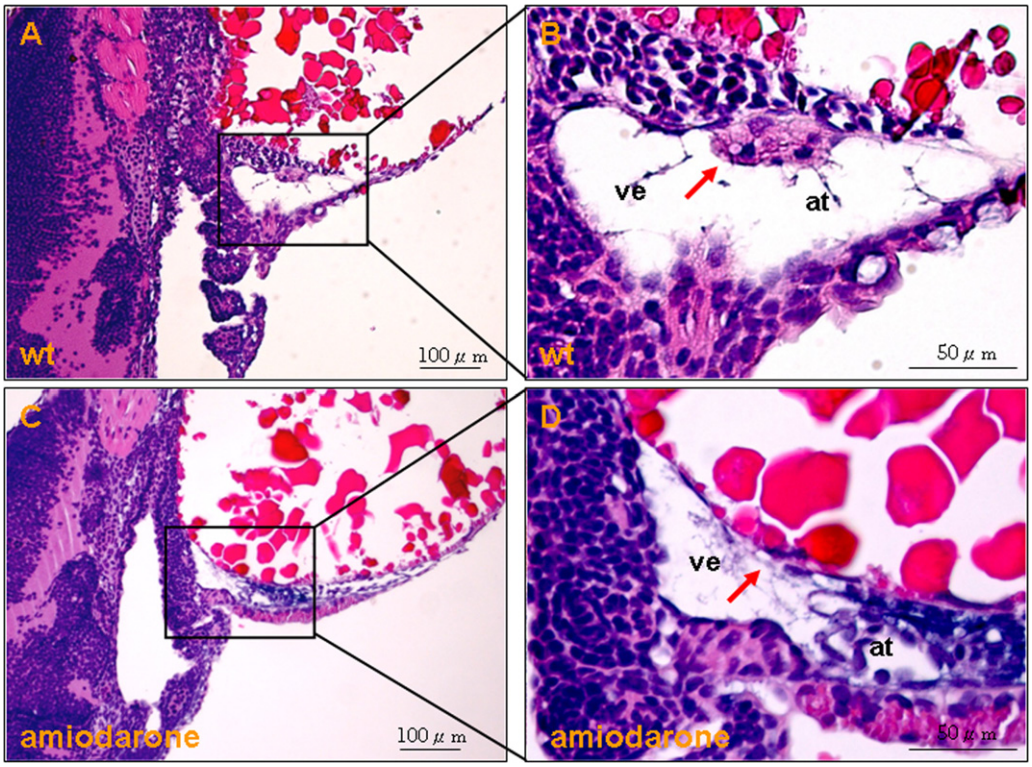

Fig. 2. Amiodarone caused developmental abnormality of cardiac valves in zebrafish embryos. To observe cardiac valve development, zebrafish embryos at 72 hpf were subjected to paraffin sectioning followed by H&E staining. (A and B), the heart of wild-type zebrafish embryos underwent invagination, and the superior endocardial cushion-forming region (ECFR) started to form endocardial cushion (arrow). (C and D) Embryos treated with 15 μM Amiodarone from 12–72 hpf displayed neither invagination progression nor endocardial cushion formation (arrow). ve, ventricle; at, atrium.

aided by paraffin sectioning and H&E staining. We observed that valve development was repressed in 72-hpf embryos treated with 15 μM Amiodarone during 12–72 hpf. Next, we focused our attention on the specification (36–55 hpf) and invagination (after 55 hpf) stages (Supplementary Fig. 1). At 72 hpf, the wild-type embryos showed the expression of *versican* at the cardiac AVC (Fig. 5A), and sectioning results revealed the existence of an endocardial cushion, the precursor structure of valve formation at the intersection of ventricle and atrium (Fig. 5E). However, 72-hpf embryos treated with 15 μM Amiodarone during 12–72 hpf showed that *versican* was ectopically overexpressed at the AVC (Fig. 5B), and no endocardial cushion at AVC was observed (Fig. 5F). In addition, 72-hpf embryos treated with Amiodarone at the specification stage showed that *versican* was ectopically expressed at the cardiac region (Fig. 5C), and no endocardial cushion structure was observed (Fig. 5G). Similarly, at the invagination stage, *versican* ectopic overexpression was observed in the 72-hpf embryos treated with Amiodarone (Fig. 5D), and no endocardial cushion structure was found (Fig. 5H). In summary, our results demonstrated that embryos treated with 15 μM Amiodarone during 12–72 hpf, including both specification and invagination stages, ectopically overexpressed *versican* in the cardiac region with no formation of the endocardial cushion. These lines of evidence suggest that Amiodarone treatment causes heart valve defect at both specification and invagination stages.

3.7. Amiodarone treatment increases cdh5 expression at zebrafish cardiac AVC

Cdh5 is an adhesion molecule expressed in endocardial cells [24], and decreased expression of *cdh5* promotes EMT [25]. To clarify whether Amiodarone affects endocardial cells during invagination, we applied WISH to detect the expression of *cdh5*, an EMT-related gene, at AVC in embryos treated with 15 μM Amiodarone during 12–72 hpf, including both the specification and invagination stages. The results showed that *cdh5* expressed restrictedly at AVC in the untreated embryos at 72 hpf (Fig. 6A). However, in the embryos treated with Amiodarone during 12–72 hpf, *cdh5* was overexpressed at AVC when observed at 72 hpf (Fig. 6B). When treated at both specification (Fig. 6C) and invagination (Fig. 6D) stages, *cdh5* was also overexpressed in the 72-hpf embryos. Thus, it is plausible that Amiodarone could induce the overexpression of *cdh5*, which would then inhibit endocardial cells from undergoing invagination and suppress the development of endocardial cushion as well. As further confirmation of this hypothesis, we performed Western blot analysis using Cdh5 antibody to determine whether Cdh5 protein is also increased in the Amiodarone-treated embryos. The results showed that Cdh5 protein was greatly increased in zebrafish embryos after Amiodarone treatment (Fig. 6E), suggesting that Amiodarone enhances the expression of Cdh5 protein. These lines of evidence demonstrated that Amiodarone not only induces the overexpression of *versican* but also induces the overexpression of *cdh5*, a downstream regulator of *versican* involved in EMT in zebrafish embryos.

3.8. Reduction of cdh5 translation by cdh5-MO injection rescues the valve defect caused by Amiodarone treatment

To understand the relationship between the valve defect caused by Amiodarone treatment and *cdh5* expression, *cdh5*-MO was injected into embryos to specifically inhibit the translation of *cdh5* transcripts [15,16]; (Supplementary Fig. 5C). The endocardial cushion was then observed with paraffin sectioning and H&E staining. In the untreated and uninjected embryos, the endocardial cushion was observed at AVC (Fig. 7A). In embryos injected with 1.6 ng *cdh5*-MO, the endocardial cushion was bulged at 72 hpf, indicating

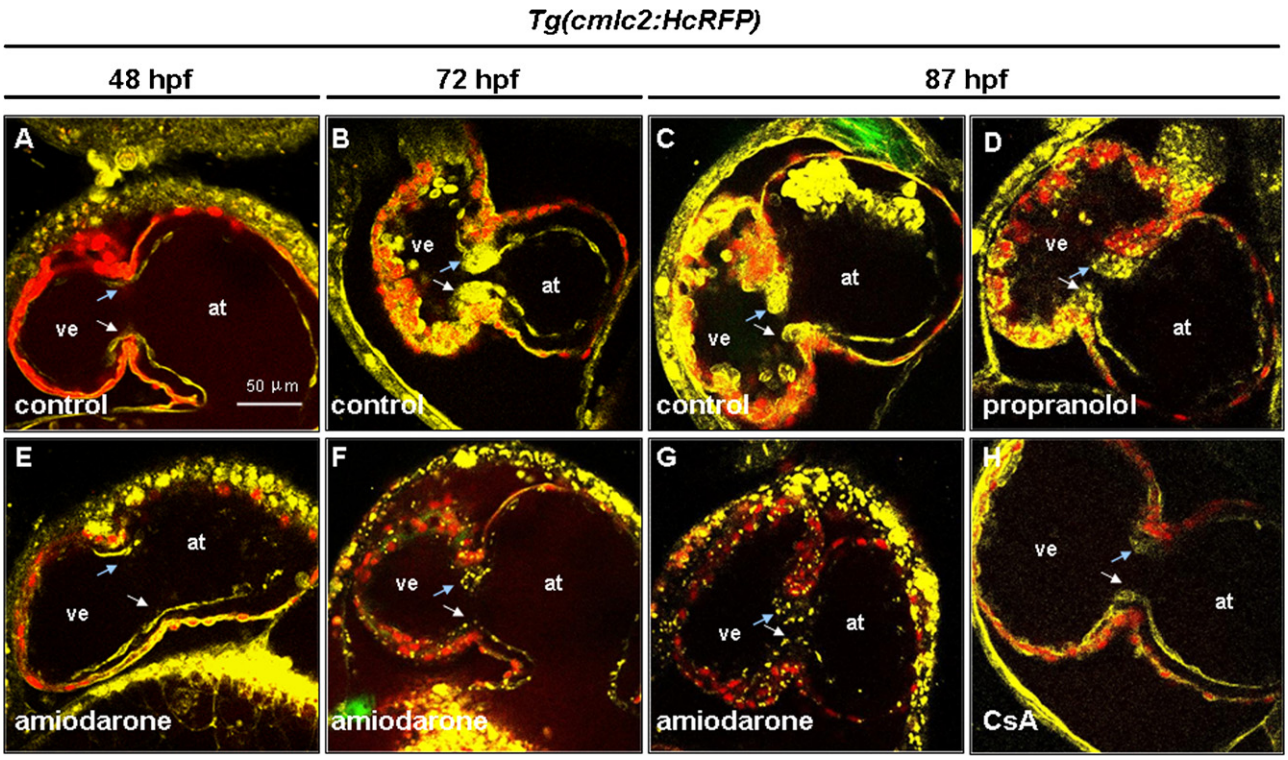

Fig. 3. *In vivo* observation of the cardiac valve defects in the Amiodarone-treated zebrafish embryos. HGM/2PF was applied to observe the valve development of transgenic zebrafish *Tg(cmlc2:HcRFP) in vivo*. RFP marked the myocardial cells, while third harmonic generation (THG, shown in yellow) marked all cells. Second harmonic generation (SHG, shown in green) marked the cardiac muscles. Valve development in zebrafish started around 36–40 hpf. Cardiac epithelial cells transformed from squamous to columnar cells (A, arrow) at 48 hpf. Columnar cells concentrated towards ECM to form the endocardial cushion through the process of invagination at 72 hpf (B, arrow). The endocardial cushion started to protrude towards the atrium and form the valve structure (C, arrow) at 87 hpf. Interestingly, the cardiac epithelial cells in embryos treated with 15 μM Amiodarone from 12–48 hpf were squamous cells (E, arrow), similar to those in non-treated embryos. However, when embryos were treated with Amiodarone from 12–72 hpf, the superior endocardial cushion-forming region (ECFR) formed a smaller and incomplete endocardial cushion (E, blue arrow), and inferior ECFR had no endocardial cushion formation (F, white arrow). The endocardial cushion was not observed to form (G, arrow), even when embryos had developed at 87 hpf, the maximal time we could observe living Amiodarone-treated embryos. Two control groups were designed: embryos treated with propranolol, an antiarrhythmic drug, from 12–87 hpf, which was followed by normal endocardial cushion development (D), and embryos treated with CsA, a known valve development inhibitor, which was not followed by endocardial cushion formation (H). ve, ventricle; at, atrium.

Table 2
The rescue experiments of Amiodarone-induced defects by knockdown of either *versican* (*versican*-MO) or *cdh5* (*cdh5*-MO).

Materials	Amount (ng)	Amiodarone treatment (μM)	No. of survival embryos among injected eggs	No. of wild-type like phenotype	No. of reduced *cdh5* expression	No. of ectopic *cdh5* expression	No. of blood regurgitation
Non-injection[a]	0	0	38/40	94.7%	0	0%	N/A
Control-MO[a]	4	0	97/112	99%	1%	0%	N/A
Control-MO[a]	8	0	122/142	99.2%	0.8%	0%	N/A
Control-MO[a]	12	0	83/93	100%	0%	0%	N/A
Control-MO[a]	16	0	106/114	98.1%	1.9%	0%	N/A
Versican-MO[a]	8	0	102/113	53.9%	46.1%*	0%	N/A
Versican-MO[a]	12	0	110/123	41.8%	58.2%*	0%	N/A
Versican-MO[a]	16	0	117/128	29.9%	70.1%**	0%	N/A
Non-injection[a]	0	15	112/175	2.7%	0%	97.3%	N/A
Versican-MO[a]	12	15	114/164	20.2%	0%	79.8%	N/A
Versican-MO[a]	16	15	113/182	41.6%	4.4%	54%**	N/A
Control-MO[b]	4	0	26/30	100%	N/A	N/A	0%
non-injection[b]	0	15	114/192	43%	N/A	N/A	57%
Versican-MO[b]	12	15	104/173	55.8%	N/A	N/A	44.2%
Versican-MO[b]	16	15	157/214	59.9%	N/A	N/A	40.1%*
cdh5-MO[b]	0.8	15	106/173	45.3%	N/A	N/A	54.7%
cdh5-MO[b]	1.2	15	118/181	52.5%	N/A	N/A	47.5%
cdh5-MO[b]	1.6	15	101/184	66.8%	N/A	N/A	33.2%*
cdh5-MO[b]	2.0	15	56/136	62.5%	N/A	N/A	37.5%

N/A, not available.
* Indicates $P < 0.05$.
** Indicates $P < 0.01$.
[a] Embryos were treated with Amiodarone starting at 12 hpf for 60 h, and then fixed by paraformaldehyde for WISH to detect *cdh5* expression.
[b] Embryos were treated with Amiodarone starting at 12 hpf for 60 h, and then observed the direction of blood flow in the heart of zebrafish embryos.

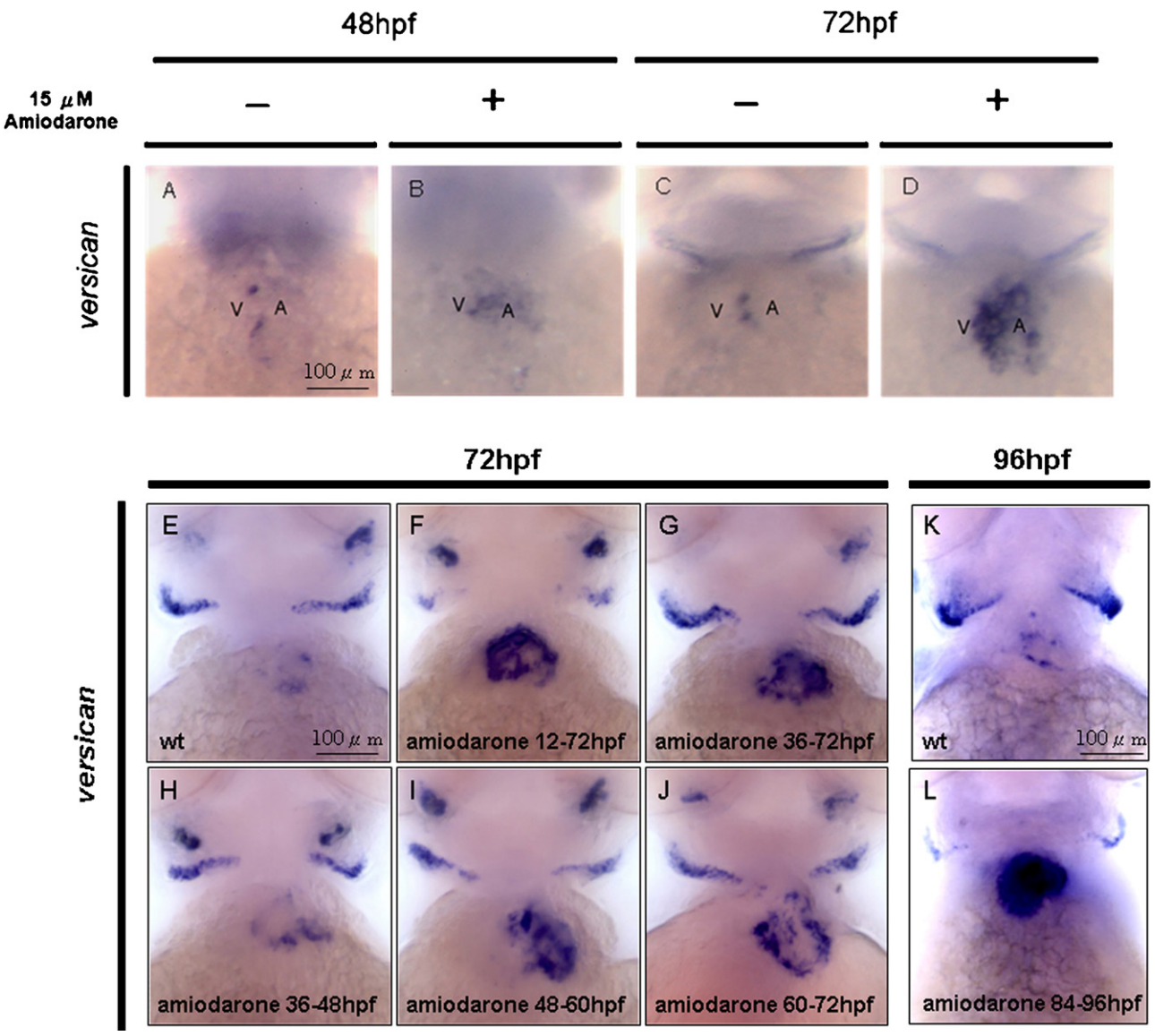

Fig. 4. Zebrafish embryos treated with Amiodarone manifested ectopic overexpression of *versican* in the cardiac region. Whole mount *in situ* hybridization was applied to indicate the expression of *versican* (A)–(D), which expressed at the intersection of ventricle and atrium, i.e. the AVC where the valves normally form. At 48 hpf, *versican* was ectopically overexpressed in the cardiac region by Amiodarone treatment, as indicated by comparing embryos without (A and C) and with (B and D) 15 μM Amiodarone treatment during 12–48 hpf. At 72 hpf, the ectopic expression of *versican* was also observed in the cardiac region, as shown by comparing embryos without (C) and with (D) 15 μM Amiodarone treatment during 12–72 hpf. The ectopic overexpression of *versican* at AVC was observed irrespective of the embryonic stage at which Amiodarone treatment was begun (12–72 hpf (F), 36–72 hpf (G), 36–48 hpf (H), 48–60 hpf (I), or 60–72 hpf (J)). *Versican* was overexpressed ectopically at AVC, even in embryos treated with Amiodarone at 84–96 hpf, after valves had already been formed (L) (A: atrium; V: ventricle).

incomplete formation of valves (Fig. 7B). Embryos treated with 15 μM Amiodarone during 12–72 hpf without *cdh5*-MO injection showed no formation of an endocardial cushion at AVC (Fig. 7C). However, embryos treated with 15 μM Amiodarone during 12–72 hpf with 1.6 ng *cdh5*-MO injection presented a normally formed endocardial cushion at AVC (Fig. 7D). Indeed, when we injected *cdh5*-MO to reduce the overexpression of *cdh5* induced by Amiodarone treatment in embryos, we found that the valve defect caused by Amiodarone was rescued (Table 2). Thus, Amiodarone causes *cdh5* to overexpress, resulting in the failure of valve formation by inhibition of the invagination process at AVC.

3.9. Amiodarone-induced valve defect can be rescued by knockdown of either versican or cdh5 in embryos

Since *versican* and *cdh5* are both genes involved in valve development, we performed experiments to understand how *versican* and *cdh5* act together to affect valve formation in Amiodarone-treated embryos. First, we microinjected *versican*-MO to reduce the expression of *versican*, and then we examined *cdh5* expression using WISH (Table 2). Paraffin sectioning and H&E staining were used to observe the development of cardiac valves. In untreated and uninjected 72-hpf embryos, *cdh5* expressed at AVC (Fig. 8A), and an endocardial cushion was observed by sectioning and H&E staining (Fig. 8E). Embryos microinjected with 16 ng *versican*-MO showed *cdh5* down-regulation at AVC at 72 hpf (Fig. 8B), resulting in an abnormally bulged endocardial cushion (Fig. 8F). In the embryos treated with 15 μM Amiodarone during 12–72 hpf, *cdh5* was overexpressed at AVC at 72 hpf (Fig. 8C), resulting in the complete absence of endocardial cushion formation (Fig. 8G). In the embryos injected with 16 ng *versican*-MO and also treated with 15 μM Amiodarone during 12–72 hpf, the expression of *cdh5* was reduced, compared to embryos treated with Amiodarone alone (Fig. 8C vs. D; Table 2). In addition, *cdh5* expression in these embryos

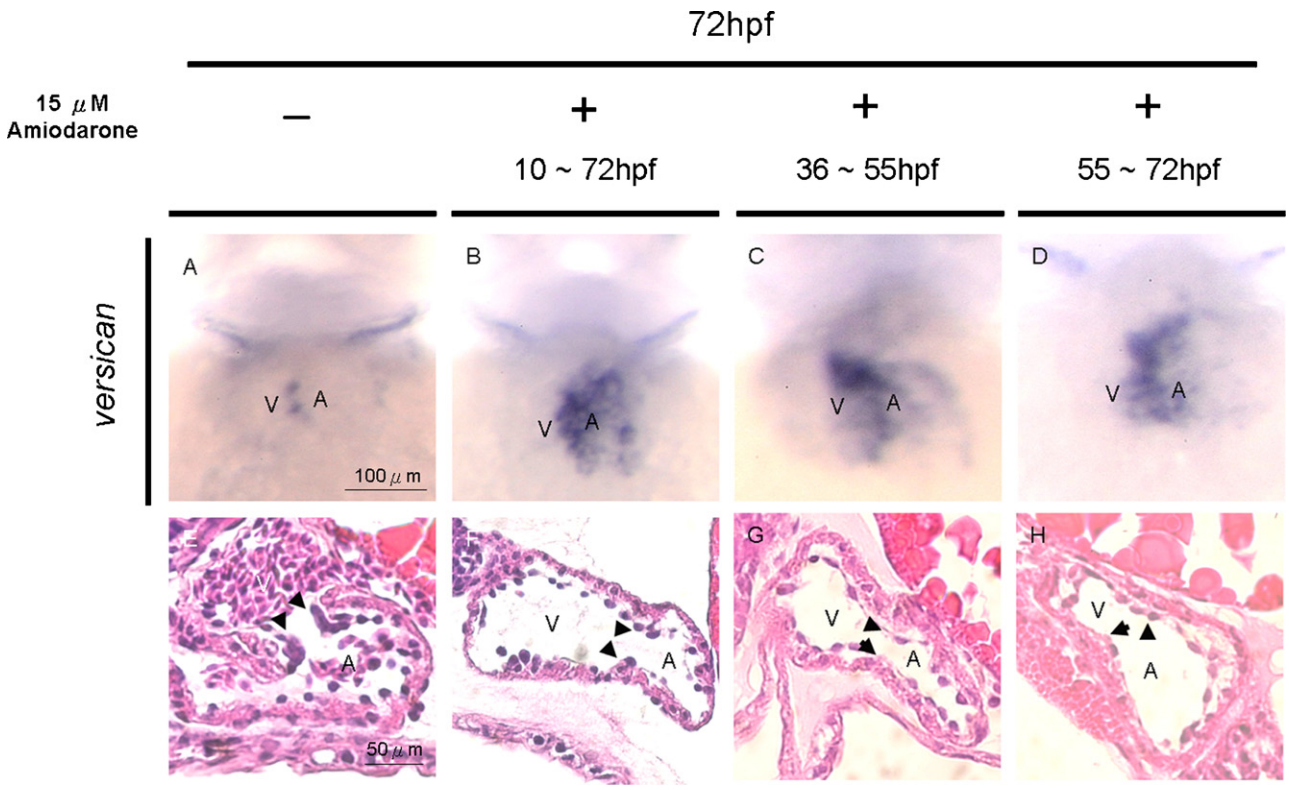

Fig. 5. *Versican* expression and endocardial cushion development in zebrafish embryos. Amiodarone-treated zebrafish embryos during the developmental stages of specification and invagination demonstrated ectopic overexpression of *versican* in the cardiac region and hypoplasia of endocardial cushion. After treating the embryos with 15 μM Amiodarone at these two stages, the expression of *versican* at 72 hpf (A)–(D) was observed with whole mount *in situ* hybridization, and the developmental status of endocardial cushion (E)–(F) was monitored with paraffin sectioning and H&E staining. In embryos without treatment, *versican* was expressed precisely at the AVC (A), and the endocardial cushion was developed normally (E, arrow). Long-term treatment of Amiodarone caused ectopic overexpression of *versican* in the cardiac region (B), and the development of endocardial cushion was suppressed (F, arrow). Amiodarone treatment during 36–55 hpf, which is the stage when endocardial cells start the specification process, also resulted in the ectopic overexpression of *versican* (C) and the repression of endocardial cushion development (G, arrow). Amiodarone treatment after 55 hpf, which starts the invagination process upon migration of endocardial cells, also resulted in the ectopic overexpression of *versican* (D) and the repression of endocardial cushion development (H, arrow) (A: atrium; V: ventricle).

was similar to wild-type embryos (Fig. 8A vs. D), and the endocardial cushion was also observed (Fig. 8H). These results demonstrate that the down-regulation of *versican* could rescue the valve defect caused by overexpression of *cdh5* at AVC, which was initially caused by Amiodarone treatment. Based on this evidence, we can postulate that Amiodarone-induced valve defect results from the ectopic overexpression of not only *versican*, but also *cdh5*, which is the downstream effector of *versican*. This finding suggests, in turn, that *versican* might positively regulate the expression of *cdh5*, thus influencing the development of endocardial cushion.

During the knockdown experiments of injecting either *versican*-MO or *cdh5*-MO, we also injected in parallel with a standard control-MO to serve as a negative control. Similar to wild-type group, no defective phenotypes were found in embryos injected with control-MO (Supplementary Fig.5A vs. B). The defective phenotypes induced by either *versican*-MO or *cdh5*-MO were dosage dependent, and injection of either *versican*-MO or *cdh5*-MO could effectively rescue the valve defects induced by Amiodarone treatment, proving that *versican*-MO and *cdh5*-MO we used are specific.

3.10. Embryos treated with a potassium channel blocker that mimics an ectopic overexpression of versican

The pharmacological mechanisms of Amiodarone are complex. Specifically, Amiodarone blocks the potassium, sodium and calcium channels and a beta-adrenergic receptor [24]. To determine which pharmacological mechanism is involved in Amiodarone-induced cardiac valve defect in zebrafish embryos, we treated zebrafish embryos with the following four drugs during 12–48 hpf and observed the expression of *versican* at 48 hpf: 4-Amiopyridine as a potassium channel blocker, Lidocaine as a sodium channel blocker, Nifedipine as a calcium channel blocker, and Propranolol as a beta-adrenergic receptor blocker. Similar to untreated embryos, embryos treated with Lidocaine, Nifedipine, and Propranolol (Supplementary Figs. 6C–E) showed a focally expressed pattern of *versican* at the AVC (Supplementary Fig. 6A). However, the 4-Amiopyridine-treated embryos displayed ectopic overexpression of *versican* at the AVC (Supplementary Fig. 6B), which was similar to the embryos treated with Amiodarone (Fig. 4B vs. Supplementary Fig. 6B). Since the potassium channel blocker caused an ectopic overexpression of *versican*, it is suggested that Amiodarone could perturb valve development-related signal transduction by blocking the flow of potassium, which, in turn, would suppress cardiac valve development in zebrafish embryos.

4. Discussion

4.1. Valve defect caused by Amiodarone is specific and unrelated to toxicity

We analyzed heart defects in embryos treated with various concentrations of Amiodarone. As shown in Table 1, compared to control group, no increase in heart defects was observed in embryos treated with lower concentrations of Amiodarone, such as 3 μM and 6 μM for 36 and 60 h. However, the occurrence percentages of pericardiac edema and blood regurgitation were dramatically increased

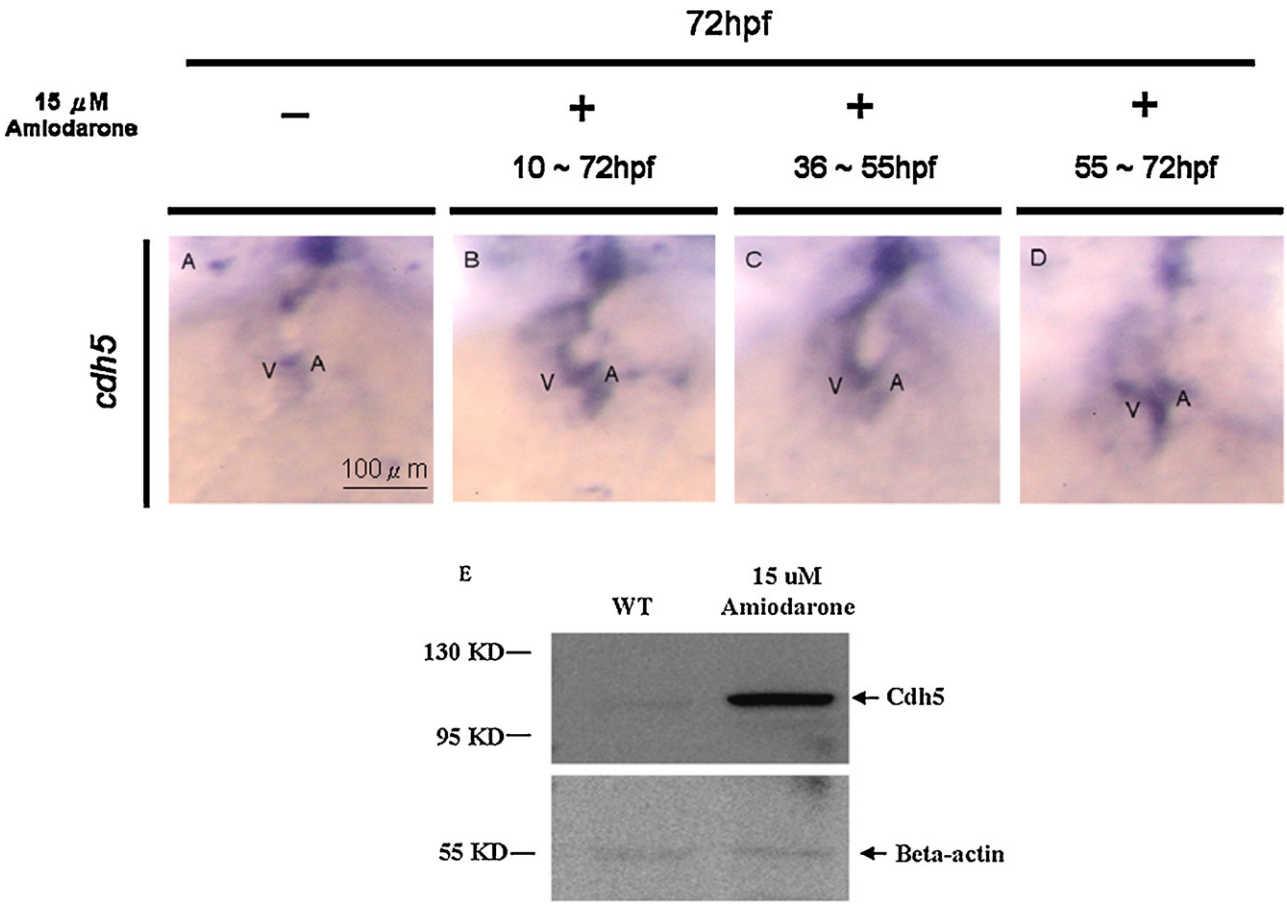

Fig. 6. Amiodarone treatment at the specification and invagination stages, respectively, resulted in *cdh5* overexpression at cardiac AVC. The expression of *cdh5*, an EMT inhibition gene, in embryos treated with 15 μM Amiodarone was observed at 72 hpf. The embryos without treatment showed normal *cdh5* expression at AVC (A), whereas those with long-term Amiodarone treatment showed overexpression of *cdh5* at AVC (B). In addition, embryos treated with Amiodarone at the specification stage only showed overexpression of *cdh5* (C), which was the same as the embryos treated only at the invagination stage (D). Western blot analysis of Cdh5 protein extracted from the zebrafish embryos with or without Amiodarone treatment (E). A: atrium; V: ventricle.

if embryos were treated with higher concentrations of Amiodarone, such as 9 μM, 12 μM and 15 μM for 36 and 60 h. The occurrence percentages of pericardiac edema and tail shrinkage were positively proportional with the treated concentration of Amiodarone. The rate of heartbeat was also greatly reduced and looping of the developing heart was incomplete in embryos treated with 15 μM Amiodarone.

Moreover, as shown in Supplementary Table 1, embryos were treated with 15 μM Amiodarone during 22–34, 34–46 and 46–48 hpf and then examined for blood regurgitation both at 48 hpf

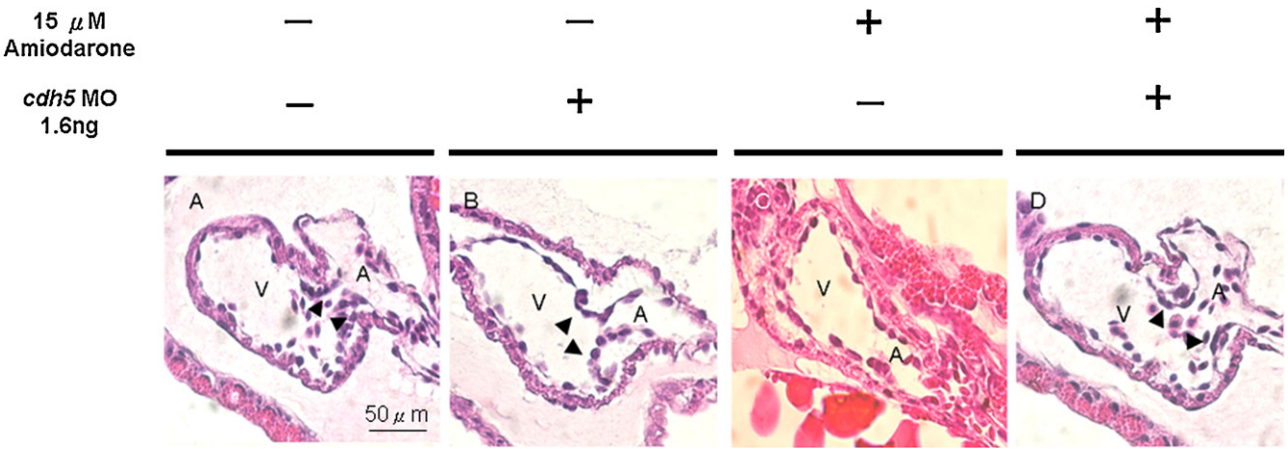

Fig. 7. Reduction of Cdh5 expression by *cdh5*-MO microinjection promoted valve development of zebrafish embryos and was able to rescue the hypoplasia of valve caused by Amiodarone treatment. Paraffin sectioning and H&E staining were applied to observe the development of endocardial cushion. In embryos absent treatment with Amiodarone and 1.6 ng *cdh5*-MO microinjection, endocardial cushion formation was observed at AVC, the intersection of ventricle and atrium (A, arrow). However, after microinjection with 1.6 ng *cdh5*-MO, the endocardial cushion bulged abnormally (B, arrow). Amiodarone treatment at 12–72 hpf resulted in inhibition of endocardial cushion development (C, arrow). Embryos with 1.6 ng *cdh5*-MO microinjection and 12–72 hpf Amiodarone treatment showed restoration of endocardial cushion development to a normal state (D, arrow). A: atrium; V: ventricle.

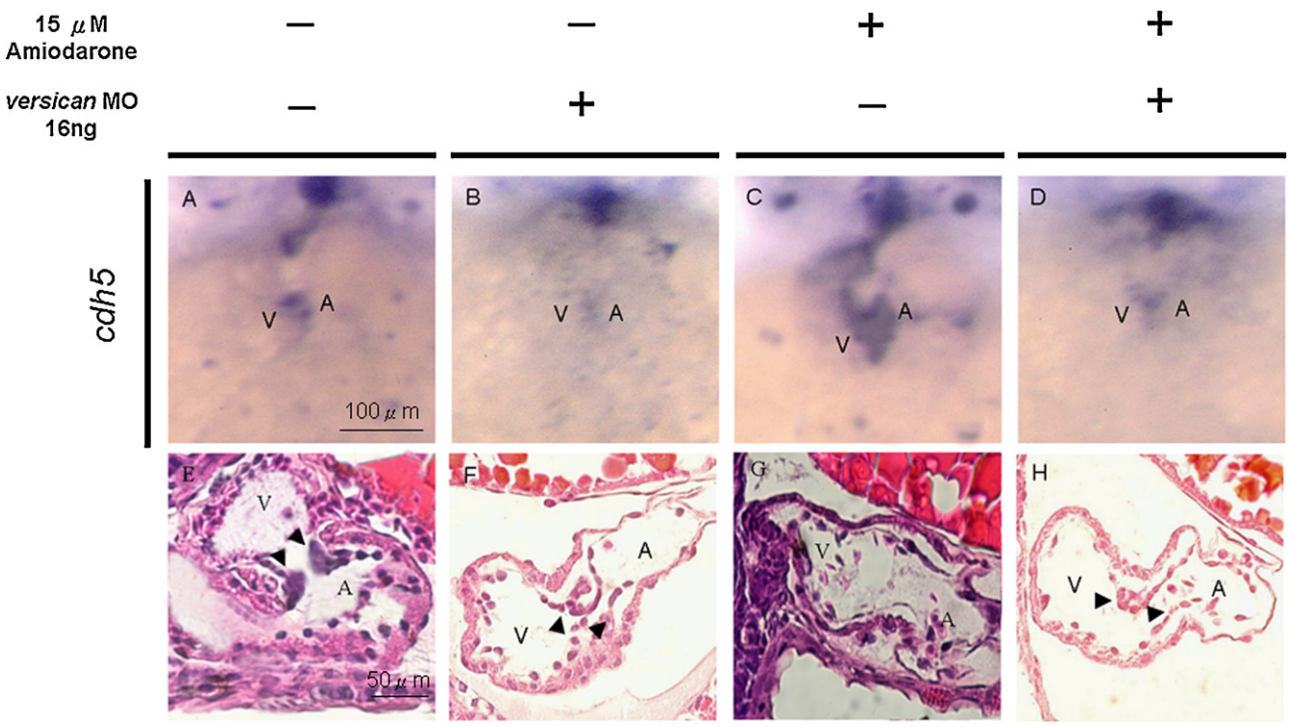

Fig. 8. Microinjection of *versican*-MO to repress *versican* expression resulted in down-regulation of *cdh5* and was able to rescue *cdh5* overexpression and malformation of values caused by Amiodarone treatment. After microinjecting 16 ng *versican*-MO at the one-cell stage, the expression of *cdh5* was observed with whole mount *in situ* hybridization (A)–(D) at 72 hpf, and the developmental status of endocardial cushion (E)–(F) was also observed with paraffin sectioning and H&E staining. In embryos without Amiodarone treatment and 16 ng *versican*-MO, *versican* was expressed at AVC precisely (A), and the structure of endocardial cushion was observed (E, arrow). Embryos microinjected with 16 ng *versican*-MO reduced the expression of *cdh5* at AVC (B), and endocardial cushion bulged abnormally (F, arrow). Embryos treated with Amiodarone at 12–72 hpf increased *cdh5* expression at AVC (C), and the development of endocardial cushion was suppressed (G, arrow). However, embryos with both 16 ng *versican*-MO microinjection and 12–72 hpf Amiodarone treatment showed levels of *cdh5* expression (D) similar to untreated embryos, and the endocardial cushion developed normally (H, arrow). A: atrium; V: ventricle.

and at 72 hpf. When we examined embryos at 48 hpf, the occurrence of blood regurgitation was dramatically increased in the embryos treated with Amiodarone either between 34 and 46 hpf or between 46 and 58 hpf, compared to control group. Such results suggested that Amiodarone causes blood regurgitation of zebrafish embryos during, but not prior to, valve formation. Moreover, we noticed that blood regurgitation was observed at 48 hpf in the one-third of embryos treated from 46 to 58 hpf, indicating that Amiodarone can rapidly act on embryos within 2 h treatment. Importantly, when Amiodarone treatment was stopped either at 46 or 58 hpf, most defective embryos displaying the blood regurgitation phenotype could be rescued by 72 hpf, indicating that heart defects induced by Amiodarone can also be abolished in the absence of Amiodarone. This line of evidence demonstrated that the heart defects caused by Amiodarone are specific and unrelated to any cytotoxicity against endocardial cells.

4.2. Valve defect caused by Amiodarone does not result from lower velocity of blood cells

Hemodynamics is a necessary component of valve development. Endocardium at the AV canal responds to blood flow-induced forces and stimulates endocardial cells to rearrange and change the genetic expression profiles [26]. After treatment with Amiodarone, embryonic heart rate was reduced, which reduces the velocity of blood flow. Therefore, to clarify whether Amiodarone-induced valve defects result from the reduction of blood flow and whether such slower blood flow is common to other antiarrhythmic drugs, we observed and compared the effect of Amiodarone and Propranalol on valve development. Propranalol is known to reduce blood flow speed. Embryos treated with Propranalol during 12–87 hpf were observed at 87 hpf with HGM/2PF. Results showed that these embryos had normal cardiac valve development (Fig. 3D). Furthermore, while hemodynamics may theoretically affect heart development, heart muscle contractions may influence valve development. To rule out this possibility, we treated embryos with either trican or knockdown of cardiac troponin T2a (*tnnt2a*) by injection of *tnnt2a*-MO into one-celled embryo to silence heartbeat. Notwithstanding these treatments, valve development proceeded normally (data not shown). Using WISH, we also found that the expression of *versican* in the AVC of *tnnt2a*-MO-injected embryos was similar to that in the AVC of untreated wild-type embryos (data not shown), suggesting that the expression of *versican* in AVC is unaffected by either the reduction in blood flow or silencing of heartbeat. Thus, we believe that valve defects do not arise from side effects of Amiodarone. Rather, the valve defects are specific to ectopic *versican* expression in the heart induced by Amiodarone. In addition, since this phenomenon specifically occurred with Amiodarone treatment, it is not an effect that can be associated with other antiarrhythmic drugs.

4.3. Amiodarone begins to influence zebrafish cardiac development during the invagination process and Amiodarone-induced valve defect results from abnormal invagination process

It was shown in this study that the heart valve defects caused by Amiodarone are not manifestations of developmental delay. Instead, by the evidence of blood regurgitation and absence of valvular structure, Amiodarone acts directly to repress zebrafish embryonic cardiac valve development. Furthermore, the fact that embryos only display defects in endocardial cushion at 72 hpf,

when invagination starts [16], suggested that Amiodarone treatment begins to affect zebrafish heart valve development during invagination.

When we treated embryos with 15 μM Amiodarone, *versican* was ectopically overexpressed at AVC during 12–72 hpf, i.e. the period covering stages of specification and invagination (Fig. 5A–D). Concurrently, the results of paraffin sectioning/H&E staining showed that zebrafish embryonic cardiac valve development was suppressed by the treatment of Amiodarone (Fig. 5E–H). Furthermore, there was no cell aggregation in the endocardial cushion region of the Amiodarone-treated embryos at 72 hpf (Fig. 3B vs. F), which, in turn, prevented endocardial cells from transforming into mobile cells to form endocardial cushion. In addition, compared to the valve defect at 72 hpf caused by Amiodarone treatment during the specification stage (Fig. 5G), treatment during the invagination stage repressed endocardial cushion development at 72 hpf (Fig. 5H). These results suggested that Amiodarone directly affects invagination to cause the defect. In further support of this inference, we found that *cdh5*, an EMT determinant gene, was overexpressed at AVC in the embryos treated with Amiodarone. Since *cdh5* is normally a repressive gene, its overexpression results in the inhibition of EMT, leading to the failure to form normal valve structures. These lines of evidence indicated that invagination is affected by Amiodarone, and thus, the endocardial cushion fails to develop properly.

4.4. Amiodarone induces ectopic overexpressions of versican and cdh5 in the AVC to inhibit valve formation

During embryogenesis, cells switch back and forth between different cellular phenotypes via mesenchymal-epithelial transition (MET) and its reverse process, EMT [27]. More specifically, in mice and chicks, the atrioventricular endocardial cells undergo EMT, as they migrate into the cardiac jelly and proliferate to form endocardial cushions. These cushions form the AV valvuloseptal complex that divides the ventricular inflow into a right and a left AV valve [28]. Formation of the atrioventricular valve of zebrafish is similar to that of higher vertebrates [22,29]. In our studies, we demonstrated that embryos treated with Amiodarone (I) fail to form cardiac valves and (II) present ectopic overexpression of *versican* and *cdh5* at AVC. We showed that knockdown of either *versican* or *cdh5* can rescue the valve defects caused by Amiodarone (Figs. 7 and 8 and Table 2). These findings suggest that the ectopic overexpression of *versican* and *cdh5* induced by Amiodarone significantly represses EMT in valves, resulting in defective phenotypes. Sheng et al. [30] reported that expression of *versican* can induce MET in NIH3T3 fibroblasts. This *in vitro* study may suggest that *versican* may repress EMT through inducing MET in the heart. Thus, we speculate that Amiodarone-induced overexpression of *versican* might seriously repress EMT at AVC. Meanwhile, Timmerman et al. [31] reported that the constitutive activation of Notch not only induces excessive endocardial EMT, but it is also required to reduce *cdh5* expression in zebrafish, indicating that *cdh5* must be reduced when EMT is processed. Thus, we speculate that Amiodarone-induced overexpression of *cdh5* might also seriously inhibit EMT at AVC.

Additionally, we knocked down *cdh5* in the Amiodarone-treated embryos and found that the valve defects caused by Amiodarone were rescued (Fig. 7). Moreover, knockdown of *versican* by a specific MO could reduce *cdh5* expression and rescue the valve defect caused by Amiodarone (Fig. 8 and Table 2). Therefore, we concluded that Amiodarone-induced ectopic expressions of *versican* and *cdh5* at AVC of embryos are specific and that Amiodarone, which induces overexpression of both *versican* and *cdh5* in the heart, leads to the repression of EMT resulting the loss of endocardial cell motility, and finally disrupts cardiac valve development.

4.5. Amiodarone treatment begun at specification still causes cardiac valve defect during the key developmental stage of invagination

In this study, treatment of 15 μM Amiodarone only at the specification stage during 36–55 hpf showed that both *versican* and *cdh5* ectopically overexpressed at zebrafish embryonic cardiac AVC at 72 hpf. The results of paraffin sectioning/H&E staining also indicated cardiac valve defect. Yet, these results were similar to those of embryos treated with Amiodarone at the invagination stage after 55 hpf. Therefore, while Amiodarone treatment affects embryos as early as the specification stage, the invagination process is still affected by the long half-life of Amiodarone. Although the process of specification occurs during 36–55 hpf, this study based its conclusions on observations recorded at 72 hpf, which, in this case, allows only a 17 h window between treatment and observation. However, the half-life of Amiodarone is 26–107 days [9]. In addition, one of the metabolites after Amiodarone is degraded, such as N-monodesethylamiodarone, still has bio-activity similar to Amiodarone [32]. Therefore, even though the drug is washed out at 55 hpf, there might still be some residual amount left in the treated embryos. Thus, the residual dosage of Amiodarone might be sufficient to continuously affect the invagination process in the embryos, even though treatment was begun at the specification stage.

4.6. Amiodarone-induced valve defect results from the overexpression of versican and cdh5 to suppress the process of invagination

In this study, zebrafish embryos treated with Amiodarone showed overexpression of *versican* at AVC. Furthermore, *cdh5*, an EMT-related gene, is also overexpressed at AVC (Fig. 6). Indeed, knockdown of *cdh5* caused the endocardial cushion to grow larger than normal, suggesting that the reduced expression of *cdh5* might result in valve enlargement (Fig. 7). On the other hand, Amiodarone treatment caused *cdh5* to overexpress at AVC (Fig. 6B), resulting in valve defect in zebrafish embryos. However, this valve defect induced by Amiodarone treatment could be rescued by injection of *cdh5*-MO to knock down *cdh5* expression. These results suggested that Amiodarone might cause zebrafish embryonic valve defect by greatly enhancing the expression of the otherwise repressive *cdh5* gene. Furthermore, Mjaatvedt et al. [33] reported that *versican* is involved in the development of cardiac chambers and valves in a mouse model system, which strengthens our hypothesis that the abnormal expressions of *cdh5* and *versican* cause valve defect in the zebrafish system by Amiodarone treatment, particularly during the critical invagination stage. To clarify the relationship between Versican and Cdh5, *versican*-MO was injected to zebrafish embryos, and the results showed that both *cdh5* and *versican* expressed in the same manner. Moreover, Versican knockdown could rescue the valve defect caused by Amiodarone (Fig. 8).

5. Conclusions

In summary, using zebrafish embryos as an experimental animal model in this study, we determined that the EC_{50} of Amiodarone is 15 μM and that the exposure time is between 34 and 58 h. We also demonstrated that the heart defects are not observed in embryos treated with lower concentrations of Amiodarone, such as 3 μM and 6 μM. However, defective hearts characterized by failure of valve formation, incomplete looping, blood regurgitation, pericardiac edema, and ventricular arrhythmia are dramatically increased

if embryos are treated with higher concentrations of Amiodarone, such as 9 μM, 12 μM and 15 μM. The rate of lethality exceeds 80% if embryos are treated with 30 μM Amiodarone. Under these conditions, women exposed to Amiodarone in early pregnancy might be at high risk for human embryonic cardiac valve defect. To decrease the rate of newborn congenital heart disease, this study might provide cardiologists and obstetricians with a reference for monitoring cardiac septum and valve development of the fetus in instances where the mother has been treated with Amiodarone 2–3 months before or during pregnancy.

Ethical statement

This study was conducted according to the guidelines recommended by the National Laboratory Animal Center. The animal use protocol was approved by the Institutional Animal Care and Use Committee of National Taiwan University (Permit Number: No. (96)-42, January-23-2008).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2011.12.008.

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