

Celecoxib Impairs Heart Development *via* Inhibiting Cyclooxygenase-2 Activity in Zebrafish Embryos

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ABSTRACT

Background: Celecoxib, a cyclooxygenase-2 inhibitor, is a commonly ingested drug that is used by some women during pregnancy. Although use of celecoxib is associated with increased cardiovascular risk in adults, its effect on fetal heart development remains unknown.

Methods: Zebrafish embryos were exposed to celecoxib or other relevant drugs from tailbud stage (10.3–72 h postfertilization). Heart looping and valve formation were examined at different developmental stages by *in vivo* confocal imaging. In addition, whole mount *in situ* hybridization was performed to examine drug-induced changes in the expression of heart valve marker genes.

Results: In celecoxib-treated zebrafish embryos, the heart failed to undergo normal looping and the heart valve was absent, causing serious blood regurgitation. Furthermore, celecoxib treatment disturbed the restricted expression of the heart valve markers bone morphogenetic protein 4 and versican—but not the cardiac chamber markers cardiac myosin light chain 2, ventricular myosin heavy chain, and atrial myosin heavy chain. These defects in heart development were markedly relieved by treatment with the cyclooxygenase-2 downstream product prostaglandin E₂, and mimicked by the cyclooxygenase-2 inhibitor NS398, implying that celecoxib-

What We Already Know about This Topic

- The selective cyclooxygenase-2 inhibitor celecoxib has been widely used in pregnant patients, yet its potential effects on fetal heart development remain unknown.

What This Article Tells Us That Is New

- Celecoxib exposure impairs heart development in zebrafish embryos by inhibiting cyclooxygenase-2 activity.

induced heart defects were caused by the inhibition of cyclooxygenase-2 activity.

Conclusions: These findings provide the first *in vivo* evidence that celecoxib exposure impairs heart development in zebrafish embryos by inhibiting cyclooxygenase-2 activity.

PREGNANT patients who suffer from systemic lupus erythematosus and other autoimmune diseases require pharmacologic control of pain and other inflammatory complications during pregnancy.¹ Cyclooxygenase inhibitors are among the most commonly prescribed drugs for controlling these complications.² Because the conventional nonselective cyclooxygenase inhibitors usually have severe adverse gastrointestinal effects, the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib has been widely used in pregnant patients because it causes minimum adverse gastrointestinal effects.^{1,3} However, recent studies have reported that celecoxib has adverse effects on the cardiovascular system in adults.^{4,5} The goal of this study is to examine whether celecoxib also affects heart development in embryos.

The zebrafish is a powerful model organism for assessing drug-induced cardiotoxicity *in vivo*.⁶ Transgenic zebrafish with vascular endothelial cells expressing fluorescent proteins allow *in vivo* imaging of the endocardium and heart valve in live embryos.⁷ Drugs diluted in medium can be absorbed by zebrafish larvae through the skin and gills. In addition, accumulating evidence has shown that drug-induced cardiotoxicity in zebrafish well resembles its effects in humans.^{6,8–10} In the current study, we used zebrafish as an animal model to

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examine the effect of celecoxib on heart development. We found that celecoxib treatment caused serious defects in heart development, including abnormality in heart looping and the absence of heart valve formation. These defects were mimicked by treatment with the COX-2 inhibitor NS398 but significantly relieved by COX-2 product prostaglandin E₂ (PGE₂). Thus, celecoxib exposure impairs embryonic heart development in zebrafish by inhibiting COX-2 activity.

Materials and Methods

Raising and Staging Zebrafish Embryo

Wild-type AB and transgenic *Tg(flk1:GFP)* zebrafish, in which vascular endothelial cells express green fluorescent protein (GFP), were obtained (Zebrafish International Resource Center, Eugene, OR) and maintained at 28°C with an automatic fish-housing system (ESEN, Beijing, China) in the National Zebrafish Resources of China (Shanghai, China). Embryos were staged as described previously.¹¹ Zebrafish handling procedures were approved by the Institute of Neuroscience, Shanghai Institutes for Biologic Sciences, Chinese Academy of Sciences. Wild-type AB embryos were used for *in situ* hybridization experiments; *Tg(flk1:GFP)* embryos were used for assessing atrioventricular valve leaflet and looping development.

Drug Treatment

Zebrafish embryos were treated with celecoxib (Pfizer Pharmaceuticals LLC, Caguas, Puerto Rico), NS398, SC560 (Cayman Chemical, Ann Arbor, MI), or acetaminophen (Sigma-Aldrich, St. Louis, MO) from the tailbud stage (10.3 h postfertilization [hpf]). Celecoxib, NS398, SC560, and acetaminophen were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to prepare stock solutions, which were then diluted to experimental concentration in embryo medium. Controls for each experiment included a negative control with embryos incubated in embryo medium and a solvent control with embryos incubated in 0.3% DMSO. Experiments were performed in triplicate. Optimal concentrations of celecoxib, NS398, SC560, and acetaminophen were 25, 60, 20 μM, and 5 mM, respectively, at which drug treatments produced obvious defects in heart development with minimal mortality before 96 hpf. At 72 hpf, drug-containing embryo medium was replaced with drug-free embryo medium.

Prostaglandins (PGs)—including PGE₂, prostaglandin I₂ (PGI₂), and prostaglandin F_{2α} (PGF_{2α})—and thromboxane A₂ receptor agonist U46619 (Cayman Chemical) were dissolved in DMSO to generate stock solutions before dilution in embryo medium at experimental concentrations. In rescue experiments, we supplemented celecoxib-exposed embryos with 1–25 μM PGE₂, PGF_{2α}, or PGI₂, and 1–10 μM U46619. Embryos were coincubated with celecoxib and PGE₂, PGF_{2α}, PGI₂, or U46619 from the tailbud stage to 72

hpf. Embryos were also treated with PGE₂, PGF_{2α}, PGI₂, or U46619 alone as negative controls.

Assessment of Blood Regurgitation and Heart Looping

To assess blood regurgitation, live embryos were mounted in 1% low melting point agarose on ventral view and imaged using a microscope equipped with a high-speed motion scope camera (NeuroLucida E600FN; Nikon Corporation, Tokyo, Japan). For analysis of celecoxib-induced heart looping defects, embryos were positioned and imaged as described above. Images were captured to measure the angle between the midline and atrioventricular canal with Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). The position of the atrioventricular canal shifted slightly during heartbeat, introducing errors in the measurement of the angle. To reduce this error, three images—which correspond to the early phase, metaphase, and telophase of atrium contraction, respectively—were used to obtain a mean angle for each embryo.

Whole Mount In Situ Hybridization

Digoxigenin-labeled probes (Roche Applied Science, Mannheim, Germany) for cardiac myosin light chain 2, ventricular myosin heavy chain, atrial myosin heavy chain, bone morphogenetic protein 4 (BMP4), and versican were synthesized using linearized plasmids. Whole mount *in situ* hybridization experiments were performed as described previously.¹²

In Vivo Confocal Imaging

To examine heart valve development, *Tg(flk1:GFP)* zebrafish embryos were mounted in 1.0% low melting point agarose with a ventral view for imaging.¹² Confocal imaging was performed at 25°C (FV1000-MPE; Olympus Corporation, Tokyo, Japan). Bidirectional confocal scans were captured at 6–10 frames/s. Time-series imaging was initiated at a random time in the cardiac cycle with 300–600 frames per embryo.

Statistical Analysis

For normally distributed data (the angle of heart looping), statistical analysis was performed (Prism 4; GraphPad Software, San Diego, CA; SPSS version 13.0; SPSS Science Software, Chicago, IL) using an unpaired two-tailed Student *t* test between two groups and one-way ANOVA plus Newman–Keuls multiple comparison test between more than two groups. For data that were not normally distributed (*e.g.*, heart rate), Kruskal–Wallis test plus Dunn's posttest was performed. For categorical variables (*e.g.*, incidence of pericardial edema, valve formation), chi-square test was performed. Statistical methods are specified in each figure legend. Summary data are represented as mean ± SD. Differences between groups were considered to be significant with a *P* value of less than 0.05.

Results

Pericardial Edema and Decreased Heart Rate

To examine the effect of celecoxib on heart development, we exposed zebrafish embryos to the drug from 10.3 to 72 hpf at concentrations of 10, 25, and 40 μM . Celecoxib-induced mortality was exhibited in a dose-dependent manner. Supplemental Digital Content 1 presents mortality rates of 48- and 72-hpf zebrafish embryos treated with celecoxib at different concentrations, <http://links.lww.com/ALN/A670>. All embryos treated with 40 μM celecoxib were dead by 72 hpf. In contrast, embryos treated with 10 μM celecoxib showed no obvious drug-induced effects in gross morphology or mortality.

We then exposed embryos to celecoxib at 10, 17.5, 25, and 32.5 μM , examining the drug's effects on heart development. Celecoxib-treated embryos exhibited serious pericardial edema and low heart rate compared with control embryos at 48 (Supplemental Digital Content 1, <http://links.lww.com/ALN/A670>) and 72 hpf (fig. 1). At 72 hpf, the incidence of pericardial edema was 13.4, 23.9, 61.9, 89.2, and 98.4% in the control, 10, 17.5, 25, and 32.5 μM celecoxib groups, respectively (fig. 1C). In addition, heart rates for these study groups were, respectively, 168.5 ± 11.9 , 136.4 ± 21.2 , 99.4 ± 17.4 , 72.4 ± 14.8 , and 38.5 ± 10.4 per minute (fig. 1D).

A similar dosage of celecoxib was used in a previous zebrafish study,¹³ in which 20 μM celecoxib reduced the number of hematopoietic stem cells. These dosages may be clinically relevant because serum concentration of celecoxib and other COX-2 inhibitors in patients ranges from 20 to 100 μM .^{14,15}

Defects in Heart Looping

During zebrafish development, the initially midline-straight embryonic heart tube transforms into a helically wound loop that subsequently loops to the right. This process is called heart looping and is conserved in vertebrates.¹⁶ Therefore, we next examined whether celecoxib treatment affects heart looping in zebrafish larvae.

Heart configuration was clearly observed by high-resolution microscopy. Supplemental Digital Content 1 demonstrates heart configuration in DMSO and celecoxib-treated zebrafish embryos, <http://links.lww.com/ALN/A670>. We found that celecoxib-treated embryos failed to undergo heart looping (fig. 2). By 72 hpf, control embryos had completed the process of heart looping, with the atrium and ventricle transferring to the left and right of the midline, respectively (fig. 2A). In celecoxib-treated embryos, the atrium and ventricle remained central and linear in a tubular structure (fig. 2B).

To determine the extent to which heart looping was affected by celecoxib treatment, we measured the angle between the midline and atrioventricular canal in ventral views (figs. 2C and D). This angle provides an index of heart looping. As shown (fig. 2E), celecoxib treatment with 25 and 32.5 μM significantly decreased the angle (DMSO, $85.3 \pm 11.8^\circ$;

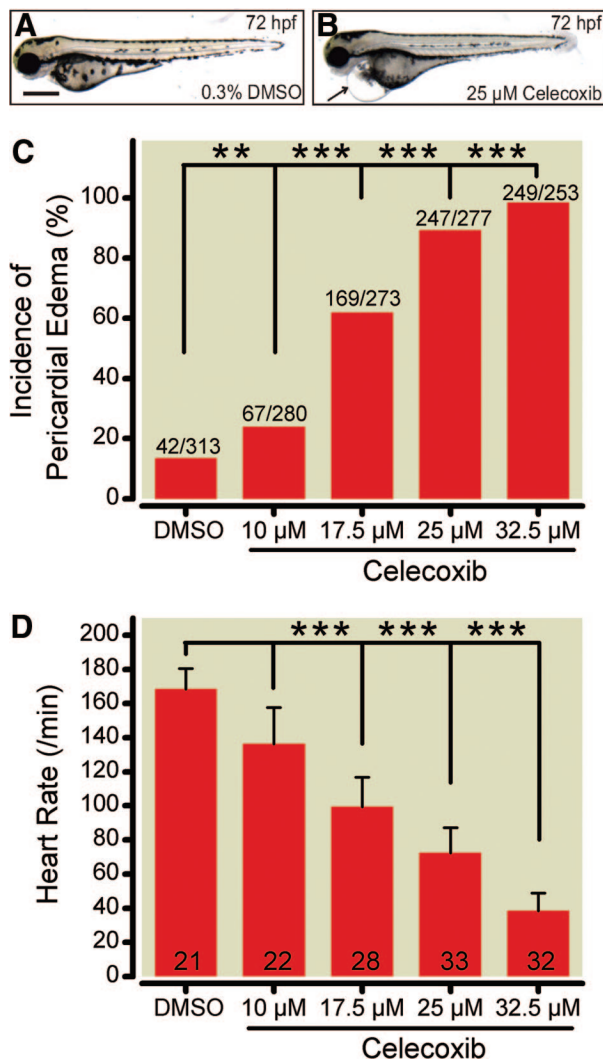


Fig. 1. Celecoxib exposure causes pericardial edema and decreases heart rate in zebrafish embryos. Experiments were performed three times. (A, B) Bright-field images show gross morphology of 72-h postfertilization embryos treated with 0.3% dimethyl sulfoxide (DMSO) or 25 μM celecoxib. Arrow indicates pericardial edema. Scale bar: 500 μm . (C) Summary of incidence of pericardial edema in 72-h postfertilization embryos as analyzed *via* chi-square test. (D) Summary of heart rate at 72-h postfertilization embryos as analyzed *via* Kruskal-Wallis test and Dunn's posttest. Results are shown as mean \pm SD. Numbers in the histogram represent the number of embryos analyzed in each group. ** $P < 0.01$. *** $P < 0.001$.

25 μM celecoxib, $38.9 \pm 15.6^\circ$; 32.5 μM celecoxib, $37.2 \pm 15.4^\circ$). These results indicate that celecoxib exposure disrupts heart looping during development.

Impaired Heart Valve Formation

The primary function of the heart valve is to prevent retrograde blood flow. To determine whether celecoxib exposure causes defects in heart valve development, we performed *in vivo* time-series confocal imaging of transgenic *Tg(flk1:GFP)*

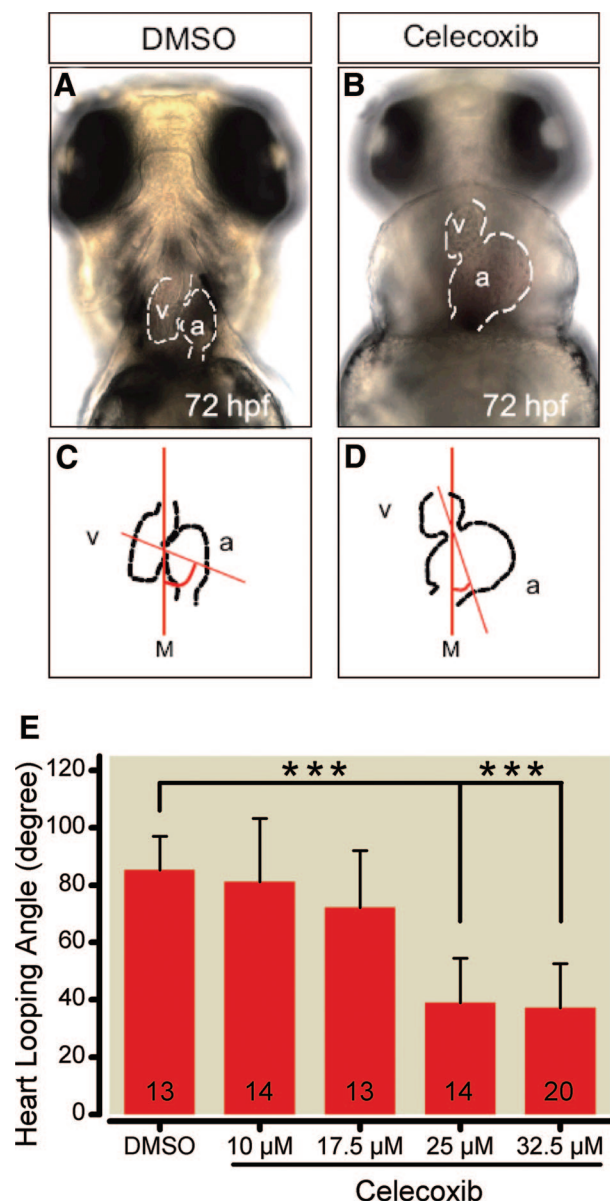


Fig. 2. Celecoxib exposure causes abnormal heart looping. (A, B) Bright-field images show (ventral view) the morphology of the atrium (a) and ventricle (v) in 72-h postfertilization zebrafish embryos treated with 0.3% dimethyl sulfoxide (DMSO) or 25 μ M celecoxib. Dashed white lines outline the heart. (C, D) Schematic shows the angle between the midline and atrioventricular canal. M, midline. (E) Summary of the angle between the midline and atrioventricular canal in 72-h postfertilization embryos as analyzed by one-way ANOVA plus Newman-Keuls multiple comparison test. Results are shown as mean \pm SD. Numbers in the histogram represent the number of embryos analyzed in each group. *** $P < 0.001$.

zebrafish larvae, which permitted visualization of the endocardium and valve structure in intact zebrafish embryos.⁷

At 85 hpf, the heart valve in control embryos was clearly observed at the atrioventricular region, whereas there was only a single sheet of endocardial cells at the atrioventricular junction in celecoxib-treated embryos. Supplemental Digital

Content 1 presents heart valve morphology in control and 25- μ M celecoxib-treated embryos at 85 hpf, <http://links.lww.com/ALN/A670>. To determine whether the celecoxib-induced valve absence was the result of developmental delay, we examined heart valve formation at a later stage. At 96 hpf, the heart valve leaflet in control embryos was well developed (fig. 3A), whereas the heart valve in 25- μ M celecoxib-treated embryos was absent at the atrioventricular junction (fig. 3B). This defect was more readily observed by high-speed confocal scanning during the heart-beat. Although the valve leaflet in control embryos was open or closed during heart contraction or relaxation, respectively, no valve leaflet was observed at the atrioventricular boundary in celecoxib-treated embryos (figs. 3D and E).

The percentages of heart valve formation were 90.6, 82.5, 49.7, and 16.3% in the control, 10-, 17.5-, and 25- μ M celecoxib-treated groups, respectively (fig. 3C). These results suggest that celecoxib treatment prevents heart valve formation.

To further examine whether celecoxib exposure causes abnormal blood flow, we used video microscopy to monitor hemodynamics. At 96 hpf, the valve leaflet in control embryos effectively prevented blood regurgitation at the atrioventricular junction. Supplemental Digital Content 2 demonstrates normal blood flow in control embryos, <http://links.lww.com/ALN/A671>. Only 7% (2 of 28) control embryos exhibited blood regurgitation. However, there was serious blood regurgitation in 90% (35 of 39) celecoxib-treated embryos, consistent with the observed absence of heart valve. Supplemental Digital Content 3 depicts blood regurgitation in celecoxib-treated embryos, <http://links.lww.com/ALN/A672>.

Altered Expression of Heart Valve But Not Cardiac Chamber Marker Genes

To determine how celecoxib affects heart valve formation, we performed whole mount *in situ* hybridization to examine the expression pattern of the early valve differentiation markers BMP4 and versican. Both genes are initially expressed throughout the heart.¹⁷ After 37 hpf, their expression is restricted to the valve-forming region. This restricted expression pattern is required for heart valve development.¹⁷

At 48 hpf, although the expression of BMP4 and versican was restricted to the atrioventricular junction in control embryos (figs. 4A and C), as reported previously,¹⁷ we found that, in celecoxib-treated embryos, both genes were expressed at the ventricle or atrium in addition to the atrioventricular junction (figs. 4B and D). However, it is noteworthy that the expression of versican in the otoliths was not changed by celecoxib exposure (figs. 4E and F), indicating that celecoxib-induced changes in versican expression are heart-specific. Therefore, these findings imply that celecoxib-induced malformation of the heart valve may be caused by altered expression of early differentiation markers of the heart valve.

In addition, we examined the effect of celecoxib treatment on the expression of cardiac markers. At 48 hpf, cardiac myosin

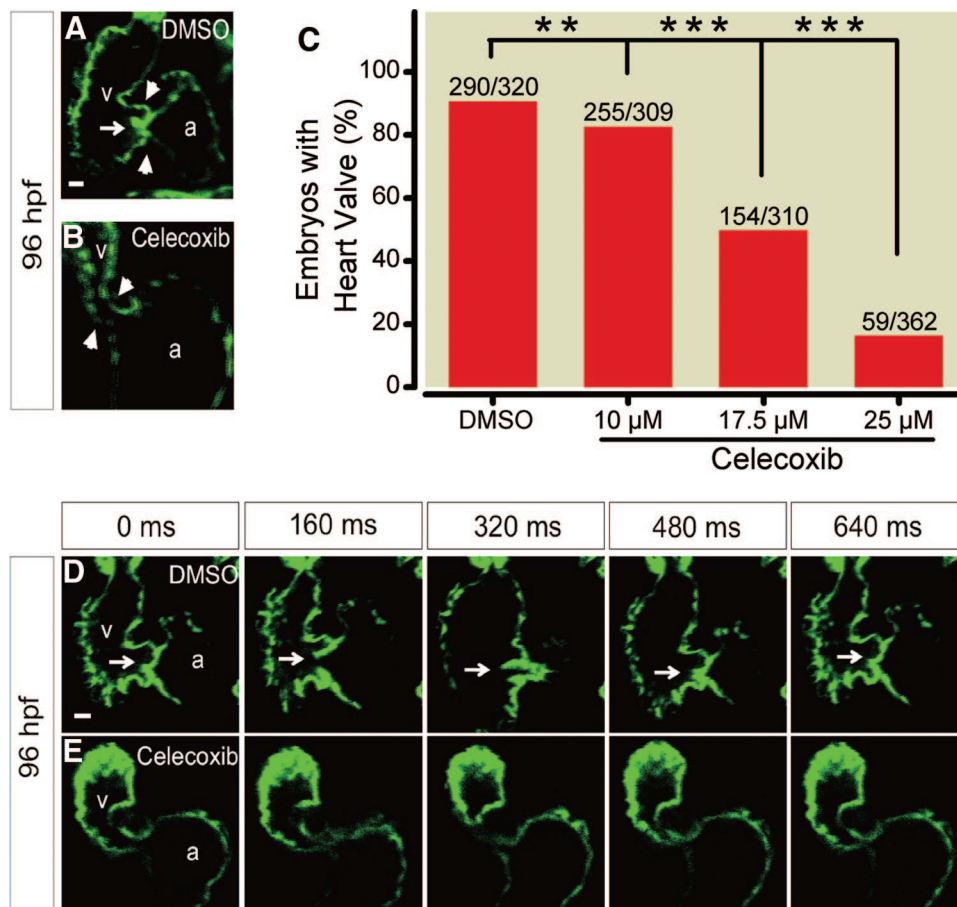


Fig. 3. Celecoxib exposure prevents heart valve formation. Experiments were performed three times. Scale bar: 20 μm . (A, B) *In vivo* confocal images of transgenic *Tg(flk1:GFP)* zebrafish, in which vascular endothelial cells express green fluorescent protein (GFP), show valve and endocardial morphology with atrium (a) and ventricle (v) in 96-h postfertilization embryos treated with 0.3% dimethyl sulfoxide (DMSO) or 25 μM celecoxib. Arrowheads point to the atrioventricular junction and an arrow indicates the heart valve. (C) Summary of heart valve formation in 96-h postfertilization embryos as analyzed by chi-square test. (D, E) Successive images during heartbeat by high-speed confocal scanning with an interval of 160 ms. ** $P < 0.01$; *** $P < 0.001$.

light chain 2 is expressed strongly in the ventricle and weakly in the atrium. In contrast, atrial- and ventricular-specific myosin heavy chain markers are expressed in the atrium and ventricle, respectively.¹⁸ We found that the expression patterns of these genes in celecoxib-treated embryos were indistinguishable from those in control embryos (fig. 5).

Celecoxib-induced Defects in Heart Development via COX-2 Inhibition

To examine whether celecoxib-induced heart defects are the result of inhibition of COX-2 activity, we treated zebrafish embryos with a mixture of celecoxib and a COX-2 product (*i.e.*, PGE₂, PGF_{2 α} , or PGI₂) or U46619. PG concentrations ranged from 1 to 25 μM ; U46619 concentration ranged from 1 to 10 μM .

We found that application of 25 μM PGE₂ significantly alleviated 25 μM celecoxib-induced defects in heart looping (figs. 6A, B, C, and D; $35.0 \pm 24.2^\circ$ in celecoxib-treated embryos *vs.* $65.5 \pm 22.6^\circ$ in cotreated embryos; $P < 0.001$) and heart valve malformation (figs. 6E,

F, G, and H; 19.9% in celecoxib-treated embryos *vs.* 62.3% in cotreated embryos; $P < 0.001$). However, coinubation with PGI₂, PGF_{2 α} , and U46619 did not relieve celecoxib-induced defects. Supplemental Digital Content 1 depicts the gross morphology of zebrafish embryos in each study group, <http://links.lww.com/ALN/A670>. These results indicate that celecoxib-induced heart defects are largely the result of decreased PGE₂ production, consistent with previous reports that PGE₂ is the predominant prostanoid effector.^{33,34}

We next examined whether exposure to another COX-2 inhibitor, NS398, could mimic the heart defects induced by celecoxib treatment. We incubated zebrafish embryos with NS398 at concentrations ranging from 30 to 100 μM . Similar to celecoxib treatment, we found that the mortality of NS398-treated embryos increased as drug concentration increased (data not shown). Exposure to 60 μM NS398 led to defects similar to those observed in celecoxib-treated embryos. NS398-treated embryos exhibited serious pericardial edema (figs. 7A, B, and C; 74.7% in NS398-treated embryos *vs.* 19% in control embryos; $P < 0.001$), abnormal heart

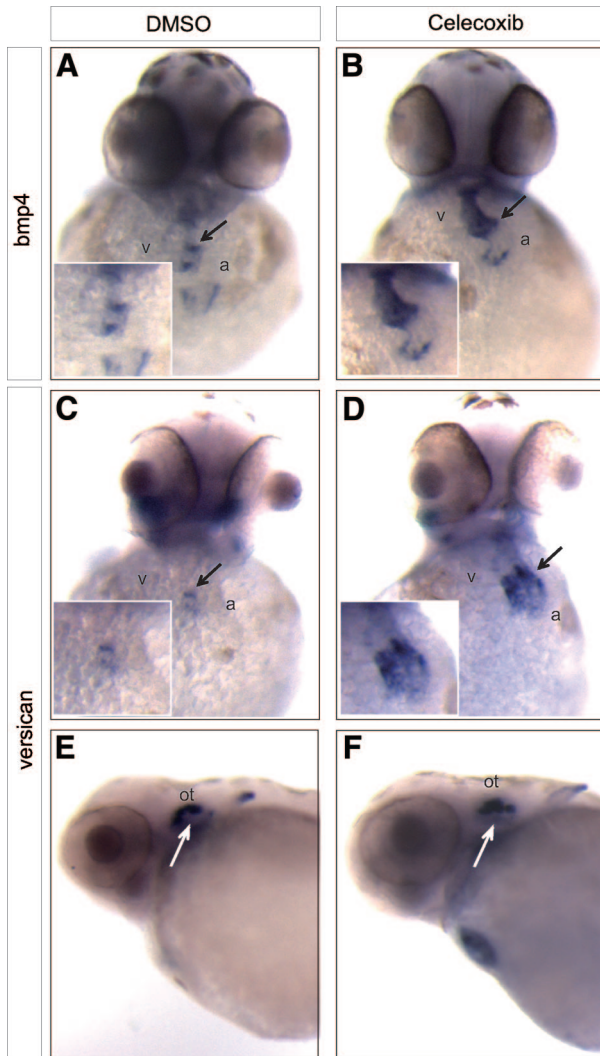


Fig. 4. Celecoxib exposure alters expression patterns of the heart valve markers bone morphogenetic protein 4 (BMP4) and versican. Whole mount *in situ* hybridization was performed in 48-h postfertilization zebrafish embryos treated with 0.3% dimethyl sulfoxide (DMSO) or 25 μ M celecoxib. Expression patterns of BMP4 (A, B) and versican (C, D) in the heart are depicted. Black arrows indicate the atrioventricular (a/v) junction. Insets show the signals in the heart area with a higher magnification. (E, F) Expression patterns of versican in the otoliths (ot), indicated by white arrows, are likewise depicted. It is noteworthy that the expression of versican in the otoliths was not changed by celecoxib exposure.

looping (figs. 7D, E, and F; $49.1 \pm 28.9^\circ$ in NS398-treated embryos *vs.* $83.9 \pm 13.7^\circ$ in control embryos; $P < 0.001$), and a low rate of heart valve formation (figs. 7G, H, and I; 36.5% in NS398-treated embryos *vs.* 87.2% in control embryos; $P < 0.001$). Consistent with heart valve malformation, we observed serious blood regurgitation in NS398-treated embryos (data not shown).

Acetaminophen, a commonly prescribed analgesic and antipyretic drug, is regularly consumed by pregnant women suffering from pain and fever.¹⁹ Recent studies^{20,21} have re-

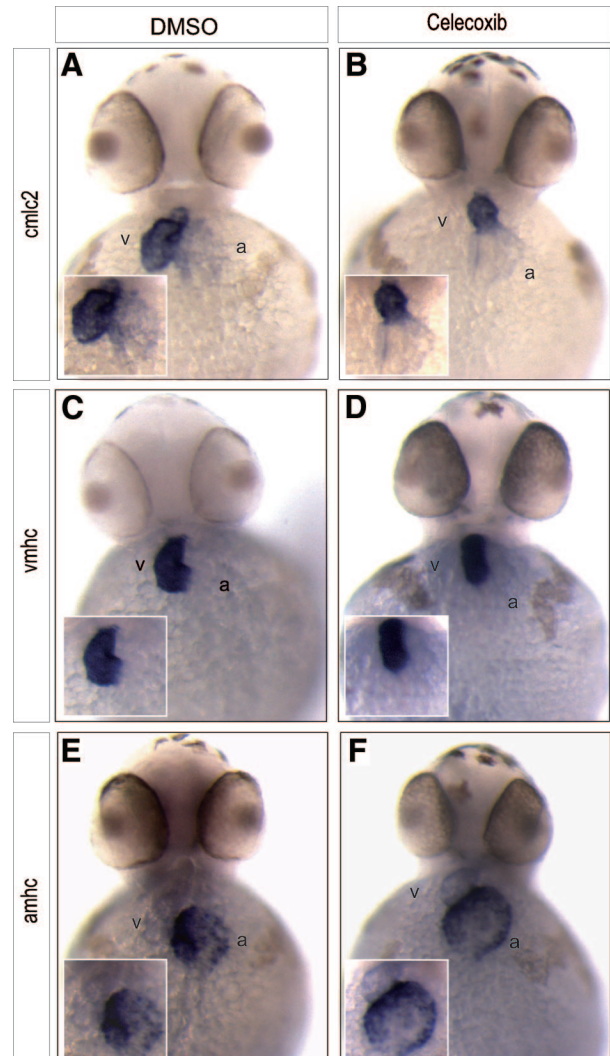


Fig. 5. Celecoxib exposure has no obvious effect on the expression of cardiac chamber markers. Whole mount *in situ* hybridization was performed in 48-h postfertilization zebrafish embryos treated with 0.3% dimethyl sulfoxide (DMSO) or 25 μ M celecoxib. Expression patterns of (A, B) the cardiac chamber marker cardiac myosin light chain 2 (cmlc2) and (C, D) the ventricle (v) marker ventricular myosin heavy chain (vmhc) are depicted, as are those of (E, F) the atrial (a) marker atrial myosin heavy chain (amhc). Insets show the signals in the heart area with a higher magnification.

ported that acetaminophen suppresses COX-2 activity and PGE₂ release, and that it may be a relatively selective COX-2 inhibitor. In addition, acetaminophen may cause cardiovascular risk in adults.^{22,23} In zebrafish embryos, acetaminophen can decrease COX-2 expression.²⁴

In addition, we found that acetaminophen exposure caused heart defects similar to those induced by celecoxib treatment, such as pericardial edema (data not shown), abnormal heart looping (figs. 8A, B, and C; $54.7 \pm 25.3^\circ$ in 5-mM acetaminophen-treated embryos *vs.* $86.3 \pm 18^\circ$ in control embryos; $P < 0.01$), and a low rate of heart valve

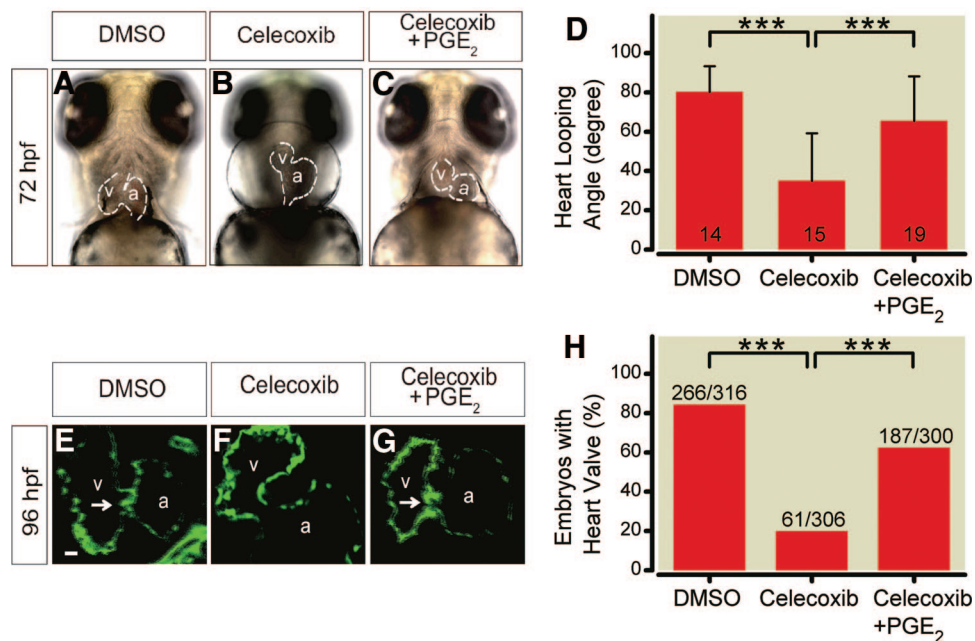


Fig. 6. Treatment with prostaglandin E₂ (PGE₂) relieves celecoxib-induced defects in heart development. Experiments were performed three times. Bright-field images showing heart looping, with atrium (a) and ventricle (v) noted, at 72 h postfertilization in (A) dimethyl sulfoxide (DMSO), (B) celecoxib-treated, and (C) celecoxib and PGE₂ cotreated embryos. (D) Summary of the angle between the midline and atrioventricular canal at 72 h postfertilization in control (n = 14), celecoxib-treated (n = 15), and celecoxib and PGE₂ cotreated (n = 19) embryos. Results are shown as mean ± SD. Heart looping angle was analyzed using a one-way ANOVA plus Newman–Keuls multiple comparison test. (E–G) Confocal images showing embryo heart valve morphology at 96 h. White arrows indicate the heart valve. Scale bar: 20 μm. (H) Summary of embryo heart valve formation at 96 h postfertilization as analyzed by chi-square test. ***P < 0.001.

formation (figs. 8D, E, and F; 38.1% in 5-mM acetaminophen-treated embryos *vs.* 88.9% in control embryos; *P* < 0.001). Taken together, these findings suggest that celecoxib-induced defects were caused by the inhibition of COX-2 activity.

Discussion

Celecoxib is a selective inhibitor of COX-2. It is one of the most commonly prescribed drugs and is used by some pregnant patients for controlling pain and other inflammatory complications. However, the adverse cardiovascular effects of celecoxib complicate its use in adults.^{4,5} Recently, there have been some concerns regarding the effects of celecoxib on fetal development. For example, clinical trials showed that taking celecoxib or other cyclooxygenase inhibitors during early pregnancy increased the risks associated with specific fetal cardiac anomalies.²⁵ Animal studies on fetal lambs showed that celecoxib has adverse effects on constriction of the fetal ductus arteriosus.²⁶ However, it remains unknown whether celecoxib exposure causes defects in heart development. In the current study, we found that celecoxib exposure seriously impaired heart development in zebrafish embryos. Celecoxib-treated embryos failed to undergo heart looping and displayed defects in heart valve formation. The restricted expression pattern of the heart valve markers BMP4 and

versican was expanded in celecoxib-treated embryos. These defects were markedly corrected by PGE₂ treatment and mimicked by NS398 exposure, suggesting that celecoxib-induced abnormalities in heart development are the result of inhibition of COX-2 activity.

COX-2 has roles in a variety of physiologic and developmental processes.^{27,28} For example, global deletion of COX-2 in mice caused reproductive and renal defects.^{29,30} Because of extra-embryonic development, zebrafish present an ideal model for investigating the functional role of cyclooxygenases and their products.^{13,31,32} Cyclooxygenases and their downstream PGs have been identified in zebrafish.^{33,34} Scherz *et al.*⁷ showed that zebrafish heart expresses COX-2. They found that exposure to the COX-2 inhibitors NS398 and CAY10404 from 56 to 72 hpf changed the shape and reduced the size of ventricle cells, causing the valve leaflet to shift ventricularly. In the current study, we observed that celecoxib or NS398 exposure affected ventricle development in zebrafish, leading to a shrunken ventricle. However, we did not observe the valve leaflet at the atrioventricular junction, the ventricle, or the atrium in celecoxib- or NS398-treated embryos. Because the zebrafish atrioventricular valve begins to form before 43 hpf,¹⁷ one explanation for this discrepancy is that we treated embryos with COX-2 inhibitors at earlier stages than previous investigators (*i.e.*, at 10.3 hpf rather than 56 hpf). It is

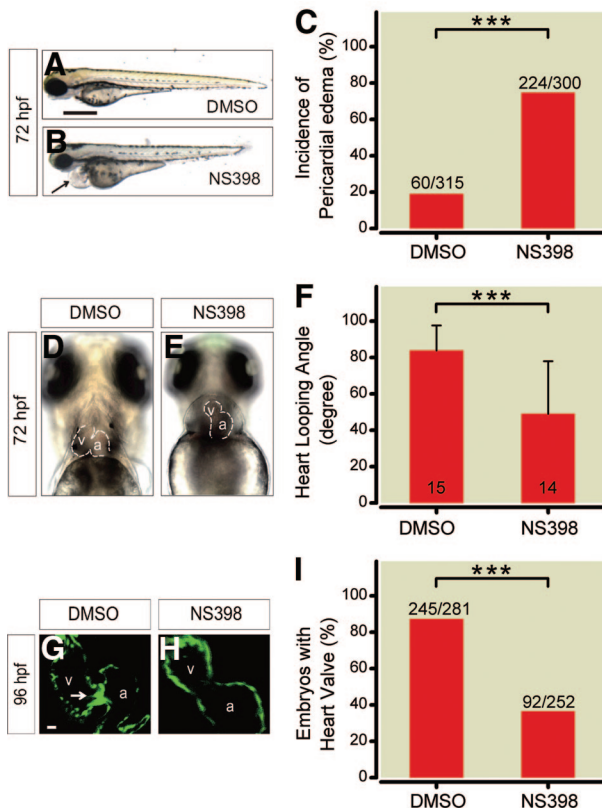


Fig. 7. NS398 exposure causes heart defects similar to those induced by celecoxib treatment. Experiments were performed three times. (A, B) Bright-field images showing the gross morphology of 72-h postfertilization (hpf) zebrafish embryos treated with dimethyl sulfoxide (DMSO) or 60 μ M NS398. Arrow indicates pericardial edema. Scale bar: 500 μ m. (C) Summary of incidence of pericardial edema in 72-hpf embryos as analyzed by chi-square test. (D, E) Bright-field images showing heart looping, with atrium (a) and ventricle (v) noted, at 72 h postfertilization in (D) dimethyl sulfoxide (DMSO) and (E) NS398-treated embryos. (F) Summary of the angle between the midline and atrioventricular canal at 72 hpf in DMSO (n = 15) and NS398-treated (n = 14) embryos. Results are shown as mean \pm SD. Heart looping angle was analyzed by unpaired two-tailed Student *t* test. (G, H) Confocal images showing heart valve morphology in 96-hpf embryos. Scale bar: 20 μ m. (I) Summary of heart valve formation in 96-hpf embryos as analyzed by chi-square test. ****P* < 0.001.

possible that COX-2 plays a critical role in atrioventricular valve formation at an early stage.

Heart valve formation requires precise cell-signaling and gene-expression patterning in the myocardium and endocardium at the atrioventricular boundary.³⁵ For example, both BMP4 and versican are involved in heart valve formation. They initially express throughout the anteroposterior extent of the heart in zebrafish. After 37 hpf, their expression is restricted to the atrioventricular valve-forming region, coincident with the time of preavalvular cushion formation.³⁶ Misexpression of these genes induces defects in heart valve formation.^{17,37} It is believed that the restricted expression pattern of BMP4 and versican is required for the cells at the

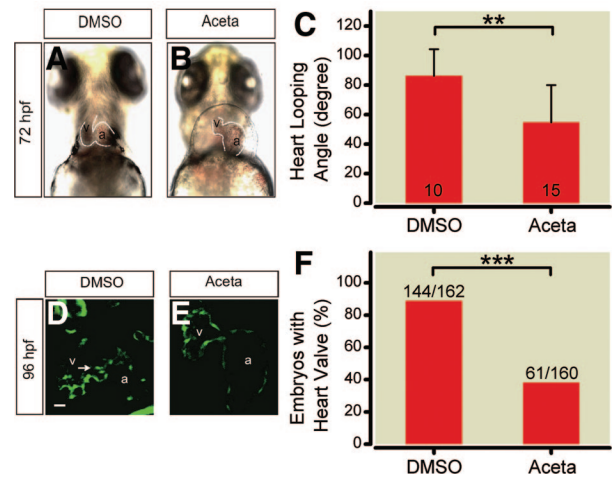


Fig. 8. Acetaminophen (Aceta) exposure causes defects in heart development of zebrafish embryos. Experiments were performed three times. Bright-field images show atrium (a) and ventricle (v) morphology at 72 h postfertilization in (A) dimethyl sulfoxide (DMSO) versus (B) 5-mM acetaminophen-treated embryos. Dashed white lines outline the heart. (C) Summary of the angle between the midline and atrioventricular canal at 72 h postfertilization in DMSO (n = 10) versus 5-mM acetaminophen-treated (n = 15) embryos. Results are shown as mean \pm SD. Heart looping angle was analyzed by unpaired two-tailed Student *t* test. (D, E) Confocal images showing the morphology of the heart valve in 96-h postfertilization embryos. Scale bar: 20 μ m. (F) Summary of heart valve formation in 96-h postfertilization embryos as analyzed by chi-square test. ***P* < 0.01. ****P* < 0.001.

border between the atrium and ventricle to adopt a tertiary cell fate during valve development. In the current study, we found that celecoxib exposure resulted in a loss of restriction in BMP4 and versican expression in the heart. In celecoxib-treated embryos, both genes were diffusely expressed in the ventricle or atrium—similar to *jekyll* and *NXT2* mutants, which have exhibited similar defects in heart valve development,^{17,36}—suggesting that celecoxib-induced heart valve defects may be the result of altered expression patterns in these genes.

In mammals, PGs play critical roles in physiologic and pathologic processes.^{27,38} For example, deletion of the PGE₂ receptor subtype 2 in mice causes arterial dilatation, salt-sensitive hypertension, and reduced fertility.³⁹ In zebrafish, PGE₂ is the primary effector of prostanoid and is regulated by cyclooxygenase-1 (COX-1) and COX-2.³⁴ PGE₂ binds to cell surface receptors and plays important roles in physiologic and developmental processes.^{13,32} Our results showed that celecoxib-induced heart defects could be attenuated by exogenous PGE₂. In contrast, application of PGF_{2 α} , PGI₂, or a manipulation of thromboxane A₂ signaling, failed to rescue celecoxib-induced defects in heart development. This result suggests that PGE₂ plays a critical role in heart development.

In the current study, we found that celecoxib-induced defects in heart development were largely corrected by PGE₂ and mimicked by the COX-2 inhibitor NS398, indicating that

COX-2 plays a major role in celecoxib-induced effects. Celecoxib is a selective COX-2 inhibitor and shows selectivity for inhibition of COX-2 375 times more than for COX-1.⁴⁰ In addition, we found that the COX-1 selective inhibitor SC560 could also cause heart developmental defects. Supplemental Digital Content 1 demonstrates SC560-induced defects in heart development, <http://links.lww.com/ALN/A670>. These results suggest that both cyclooxygenases are involved in heart development.^{7,34} Consistent with the fact that COX-1, but not COX-2, is expressed in trunk vasculature and required for vascular development in zebrafish embryos,³¹ we found that only SC560 (20 μ M), but not celecoxib (25 μ M), treatment impaired vascular development and resulted in shorten or absent intersomitic vessels. Supplemental Digital Content 1 depicts vascular development in control, celecoxib, and SC560-treated embryos, <http://links.lww.com/ALN/A670>. This finding implies that celecoxib, at the dosages used in our study, mainly affects COX-2—but not COX-1—activity.

During the past decade, numerous studies have focused on understanding inherited causes of congenital heart diseases, but the adverse effect of drug treatment on fetal heart development has received less attention. Herein, we demonstrate for the first time that celecoxib exposure affects heart development, including the heart looping and valve formation. We hope to direct attention to fetal safety in pregnant women using celecoxib.

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ANESTHESIOLOGY REFLECTIONS

The Barn by Vandam



A past Editor of ANESTHESIOLOGY, Leroy D. Vandam, M.D., certainly enjoyed creating artworks associated with an earlier Baystater, celebrated etherizer W. T. G. Morton (1819-1868). When Morton was 8 yr of age, his family home in Charlton, Massachusetts, burned to its foundation. By the time a replacement home was erected nearby (as seen in Vandam watercolors *Morton House I* and *II*), Morton's family had already moved to the Waters-Morton House, which Vandam also painted at least twice. So, unlike the buildings in Vandam's other watercolors, this weathered barn (*above*) stands on the actual birth site of Morton. Owned in 1857 by J. Lamb and in 1898 by F. F. Prenier, this 19th century English-style barn has been remodeled for gable-end access. Painted by Vandam in the 1980s, this watercolor was acquired by the Wood Library-Museum in September of 1993. (Copyright © the American Society of Anesthesiologists, Inc. This image also appears in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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