3D light-sheet assay assessing novel valproate-associated cardiotoxicity and folic acid relief in zebrafish embryogenesis

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HIGHLIGHTS

- Cardiotoxicity in zebrafish embryogenesis was assessed with light-sheet microscopy
- Valproic acid dislocates heart from outflow tract in zebrafish embryogenesis
- Light-sheet analysis quantitates VPA-associated hypoplastic heart and folate relief
- Folic acid raises cell number and heart function in early zebrafish cardiac development

ABSTRACT

Precise in vivo toxicological assays to determine the cardiotoxicity of pharmaceuticals and their waste products are essential in order to evaluate their risks to humans and the environment following industrial release. In the present study, we aimed to develop the sensitive imaging-based cardiotoxicity assay and combined 3D light-sheet microscopy with a zebrafish model to identify hidden cardiovascular anomalies induced by valproic acid (VPA) exposure. The zebrafish model is advantageous for this assessment because its embryos remain transparent. The 3D spatial localization of fluorescence-labeled cardiac cells in and around the heart using light-sheet technology revealed dislocalization of the heart from the outflow tract in two-day-old zebrafish embryos treated with 50 μM and 100 μM VPA (P < 0.01) and those embryos exposed to 20 μM VPA presented hypoplastic distal ventricles (P < 0.01). These two observed phenotypes are second heart field-derived cardiac defects. Quantitative analysis of the light-sheet imaging demonstrated that folic acid (FA) supplementation significantly increased the numbers of endocardial and myocardial cells (P < 0.05) and the accretion of second heart field-derived cardiomyocytes to the arterial pole of the outflow tract. The heart rate increased in response to the cellular changes occurring in embryonic heart development (P < 0.05). The present study disclosed the cellular mechanism underlying the role of FA in spontaneous cellular changes in cardiogenesis and in VPA-associated cardiotoxicity. The 3D light-sheet assay may be the next-generation test to evaluate the risks of previously undetected pharmaceutical and environmental cardiotoxicities in both humans and animals.

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1. Introduction

The release of chemicals from pharmaceutical fabrication into aquatic ecosystems affects human and animal life (Cunningham et al., 2009; Monteiro and Boxall, 2010; Boxall et al., 2012; Brodin et al., 2013; Babic et al., 2018). Consequently, risk management requires sophisticated assessment methods to disclose hidden toxicity. It is important to understand the developmental toxicities
of these anthropogenic chemicals and to evaluate the unseen threats they may pose to human health and the aquatic ecosystem.

Prenatal use of antiepileptic is common but is associated with increased risks of congenital malformations (Meadow, 1968; Tomson and Battino, 2012; Pennell, 2016), impaired cognitive and behavioral development (Christensen et al., 2013; Meador et al., 2013), and intrauterine death (Bech et al., 2014; Tomson et al., 2015; Harden, 2016). Valproic acid (VPA) is used to control epilepsy (Genton et al., 2006; Zhu et al., 2017), bipolar disorders (Gobbi and Janiri, 2006; Smith et al., 2010), social phobia (Jessorger et al., 2007), neuropathy and migraines (Qing et al., 2008), and other neurological conditions. VPA has been tested as a neuroprotectant and in the treatment of Huntington’s and Alzheimer’s diseases (Ren et al., 2004; Qing et al., 2008; Zadori et al., 2009). However, VPA exposure during pregnancy is associated with increased incidences of neural tube defects (Ornay, 2006), autism (Markram et al., 2007; Christensen et al., 2013), craniostenosis (Lajeunie et al., 2001), skeletal malformations (Finnell et al., 2002), urogenital and limb defects (Braenn et al., 2010), and heart abnormalities (Vajda and Eadie, 2005; Ozkan et al., 2011). The frequency of these conditions and the mechanisms by which VPA induces them remain to be determined.

Zebrafish (Danio rerio) are model organisms substituting for animal experimentation. They have been used to assess acute chemical toxicity and environmental contamination. Zebrafish embryos remain transparent until the late larval stage. This trait supports unobstructed observation and imaging of morphological changes and extensive developmental characterization. VPA toxicity in zebrafish is associated with developmental delay (Gurvich et al., 2005; Selderslghs et al., 2009; Teixido et al., 2013), yolk and cardiac edema (Yu et al., 2016), and defects in brain development (Terbach et al., 2011; Cowden et al., 2012) and eye and somite formation (Selderslghs et al., 2009), cardiovascular defects (Li et al., 2009; Braenn et al., 2010), and skeletal deformities (Braenn et al., 2010).

Folic acid (FA; Vitamin B9) is not synthesized de novo in humans. Therefore, dietary FA intake or supplementation is required daily. FA participates in DNA synthesis, repair, and methylation. It reduces the risks of embryonic and fetal neural tube defects in humans (Hernandez-Diaz et al., 2000; Czeizel et al., 2013). Moreover, periconceptional FA supplementation may also reduce the risks of cardiovascular (Czeizel, 1996; Czeizel et al., 2011b; Ahmad et al., 2017; Mao et al., 2017), oral (Gildestad et al., 2015; Ebadiar et al., 2016), urinary tract (Czeizel, 1996; Czeizel et al., 2011a), and limb-reduction (Czeizel, 1996; Werler et al., 1999; Clevs et al., 2011; Ebadiar et al., 2016) defects. FA supplementation mitigates ethanol-induced pericardial edema and endocardial cushion defect (Sarmah and Mars, 2013) and VPA-induced bradycardia, pericardial edema, abnormal heart looping, and atrioventricular valve malformation (Yu et al., 2016) in zebrafish embryogenesis. FA protects against spontaneous embryonic neural tube defects. However, it remains to be established whether FA supplementation during human and animal pregnancy also protects the embryo against drug-associated neural tube defects (Patel et al., 2018).

Recent advances in imaging technologies have facilitated sensitive and informative toxicological screening using zebrafish embryos. Light-sheet microscopy (single-plane illumination microscopy; SPIM) uses a millimeter-thick laser sheet to illuminate the focal plane. This configuration minimizes sample exposure time and allows faster throughput and longer imaging protocols without compromising image quality. The extended field of view in light-sheet microscopy identifies developmentally important interactions between cells and tissues. Light-sheet microscopy has several advantages over conventional methods in the analysis of zebrafish embryonic development (Huisken and Stainier, 2007; Keller et al., 2008, 2011; Santi et al., 2009; Mertz and Kim, 2010; Truong et al., 2011; Weber and Huisken, 2011; Tomer et al., 2012; Chardes et al., 2014; Park et al., 2015).

In this study, light-sheet imaging revealed that VPA treatment physically split the embryonic heart from its outflow tract during the heart looping stage. This phenotype was not previously reported possibly because of limitations in conventional imaging technology and the use of traditional animal models. The light-sheet assay also disclosed the VPA-associated hypoplastic heart and FA relief. The results substantiated the putative role of FA supplementation against the spontaneous occurrence of hypoplastic heart. The present study demonstrates that light-sheet imaging of zebrafish cardiogenesis is a powerful assay to assess unseen cardiotoxicity of pharmaceuticals and environmental pollutants.

2. Materials and Methods

2.1. Zebrafish genetic background and maintenance

Zebrafish (Danio rerio) wild type AB and the transgenic reporter strains Tg(cmlc2:egfp) (Huang et al., 2003) and Tg(kdrf.mcherry) (Proulx et al., 2010) were maintained in saltwater at 27.5°C under a 14-h/10-h light/dark cycle. Embryos were obtained by natural spawning, raised at 28.5°C in E3 medium (5 mM NaCl, 0.33 mM MgSO4, 0.33 mM CaCl2, and 0.17 mM KCl), and staged by hours post-fertilization (hpf) or days post-fertilization (dpf) (Kimmel et al., 1995). All procedures were approved by the Animal Use and Ethics Committee of Kangwon National University, Korea (Protocol No. KW-151112-1) and in accordance with IACUC guidelines and the National Law for Laboratory Animal Experimentation.

2.2. Pharmacological treatment

Sodium valproate (Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in E3 medium (5 mM NaCl, 0.33 mM MgSO4, 0.33 mM CaCl2, and 0.17 mM KCl). Preliminary screening determined that zebrafish embryos should be treated with 0 µM, 20 µM, 50 µM, or 100 µM valproic acid (VPA). Twenty embryos per treatment were subjected to VPA in 4 mL E3 in a 35-mm dish. The exposure time was 19 h and spanned from the 50% epiboly stage (5 hpf) to the chorion stage at 24 hpf. The time interval corresponds to embryonic period from gastrulation to early organogenesis. The embryos were washed 3 times with E3 and maintained in VPA-free E3 until examination. Toxicity phenotypes were evaluated by comparing embryos treated with VPA against untreated controls.

To investigate the cellular mechanism of folic acid prevention of VPA cardiotoxicity, we supplemented embryonic VPA treatment with 0.2 µM folic acid (Sigma-Aldrich Corp., St. Louis, MO, USA) as previously described (Yu et al., 2016). Folic acid (FA) stock was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp., St. Louis, MO, USA). Embryos were treated with VPA in 1% DMSO/E3 from 5 hpf to 24 hpf and 0.2 µM FA supplementation from 5 hpf to 48 hpf. The effects of FA were evaluated by comparing the embryos treated with both VPA and FA against both controls. All experiments were repeated at least three times.

2.3. Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Thises and Thises, 2008). Briefly, antisense RNA probes were prepared with a DIG RNA labeling mix (11277073910; Sigma-Aldrich Corp., St. Louis, MO, USA). Wild type zebrafish embryos were dechorionated, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated through a graded methanol
The raw data acquired from the light-sheet imaging were processed with ZEN software v. 2.0 (Carl Zeiss AG, Oberkochen, Germany). Optical sections were merged for each region of interest (ROI).

The hybridization buffer consisted of 50% deionized formamide (Applied Biosystems, Foster City, CA, USA), 1X saline-sodium citrate (SSC) with citric acid (pH 5.0), 0.1 mg/mL heparin (Sigma-Aldrich Corp., St. Louis, MO, USA), 0.05 mg/mL tRNA (F. Hoffmann-La Roche AG, Basel, Switzerland). 5 mg/mL 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (Sigma-Aldrich Corp., St. Louis, MO, USA), and 0.1% Tween 20 (Sigma-Aldrich Corp., St. Louis, MO, USA), was added to the hybridization buffer, and hybridized at 65°C overnight.

The 3D light-sheet imaging was performed under a Lightsheet Z.1 fluorescence microscope (Olympus, Tokyo, Japan). Bright-field images were captured with an AxioCam HRc camera (Carl Zeiss AG, Oberkochen, Germany).

2.4. Live light-sheet imaging of zebrafish embryos

To visualize cardiac cells using light-sheet microscopy, we used the transgenic zebrafish line Tg(cmlc2:egfp) in which the myocardi are labeled by the expression of the green fluorescence protein (EGFP) in the myocardial cells under the cmlc2 promoter (Huang et al., 2003). We also used a Tg(kdrl:mcherry) line in which the endocardial are labeled by the expression of the mCherry fluorescence protein in the endothelial cells, including the endocardial cells, under the kdrl promoter (Proulx et al., 2010). Tg(cmlc2:egfp/kdrl:mcherry) embryos were obtained by mating the two afore-mentioned lines.

The 3D light-sheet imaging was performed under a Lighthsheat Z.1 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany; Korea Basic Science Institute Chuncheon Center), as previously described (Park et al., 2015; Eum et al., 2016). Each embryo was anesthetized in E3 containing 0.02% tricaine, embedded, and centered within glass capillaries (1.0 mm I.D.; Carl Zeiss AG, Oberkochen, Germany) filled with 1.5% low-melting point agarose in the same medium. The agarose cylinder containing the anterior portion of the embryo was extruded from the capillary. The latter was then set in the imaging chamber. The imaging chamber was filled with E3 plus 0.02% tricaine and maintained at 27°C throughout the imaging period. A Lighthsheat Z.1 fluorescence microscope with a W Plan-Apochromat 20 × 1.0 UV-VIS 4909000119 objective lens was used to collect data from the 3D imaging. EGFP and mCherry fluorescence were excited with 488 nm and 561 nm lasers, respectively. Emission was detected by 500–545 nm and 575–615 nm bandpass (BP) filters, respectively, as previously described (Park et al., 2015). The raw data acquired from the light-sheet imaging were processed with ZEN software v. 2.0 (Carl Zeiss AG, Oberkochen, Germany). Optical sections were merged for each z-stack by maximum intensity projection. The stacked sections were merged to form an image for each region of interest (ROI).

Three-dimensional images were reconstructed from optical sections and video-processed by Imaris v. 8.4 (Bitplane, Zurich, Switzerland) at the Korea Basic Science Institute Chuncheon Center.

2.5. Data and statistical analysis

Survival curves were generated in GraphPad Prism v. 5.03 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). VPA-associated embryonic death was confirmed by the absence of a heartbeat. The 95% confidence interval (CI) was calculated for instantaneous fractional survival time. Statistical analyses included an independent-samples t-test and Fisher’s exact test in SPSS for Windows v. 23.0 (IBM Corp., Armonk, NY, USA). All zebrafish data are presented as means ± SEM per embryo or phenotype. Results were considered significant when P < 0.05.

Heart looping phenotypes were grouped as normal (dextro- or D-loop: ventricular loop is curved rightward), incomplete (looping is not complete), unloop (ventricular loop is not curved), and L-loop (ventricular loop is curved leftward). Each phenotype was enumerated according to the results of the in situ hybridization of the embryos treated with VPA in the absence or presence of FA. Ten or more embryos were analyzed for each treatment and experiments were repeated three times. Data were analyzed by Fisher’s exact test.

Myocardial and endocardial cells in the cardiac chambers of VPA-treated embryos were counted in the 3D video images obtained from light-sheet microscopy of VPA-treated Tg(cmlc2:egfp/kdrl:mcherry) embryos in the absence or presence of FA at 53 hpf to quantitate VPA cardiotoxicity at the cellular level. Data were analyzed by an independent-samples t-test. Experiments were repeated at least three times per treatment.

3. Results

3.1. VPA alters myl7 RNA expression pattern in zebrafish cardiac development

To understand the detailed mechanism of developmental cardiotoxicity caused by valproic acid (VPA) exposure, the VPA-treated embryos were collected at 48 hpf and subjected to in situ hybridization using myl7 RNA probe, a cardiomyocyte differentiation marker. Heart looping was delayed or defective at 48 hpf in embryos treated with 20 μM VPA. Therefore, the embryos with looping defect were counted and the data were subjected to Fisher’s exact test. It was found that 62 ± 6% of the embryos had an incomplete loop, 36 ± 5% presented with an unloop, and 3 ± 2% displayed the L-loop (left-sided) (Fig. 1; P < 0.0001). The ventricles of embryos treated with 50 μM VPA were not inflated (Fig. 1A d; 27 ± 8%) or their myl7 mRNA expression pattern was interrupted in the middle of the heart (Fig. 1A e; 73 ± 8%). Cardiac defects in embryos treated with 100 μM VPA were relatively more severe and induced tiny amount of myl7 RNA expression (Fig. 1 A f; 68 ± 3%). Therefore, VPA altered myl7 gene expression in early cardiac development and caused abnormal heart looping and chamber formation.

3.2. 3D light-sheet microscopy reveals VPA-associated physical split of second heart field-derived structures

We investigated the cell-level mechanism of VPA-associated interruption of myl7 mRNA expression in the heart of developing embryos. To visualize cardiac cells, we used Tg(cmlc2:egfp/kdrl:mcherry) double-transgenic zebrafish, treating their embryonic progeny with VPA, and performing in vivo live imaging of the embryonic hearts under light-sheet microscopy/SPIM as described in the Materials and Methods.
Compared to the control, distal ventricle formation from second heart field progenitors was incomplete in 53-hpf embryos treated with 20 μM VPA (Fig. 2A, a,b, arrow; Supplementary Videos A, B). Therefore, VPA may cause a delay or defect in the late differentiation or migration of second heart field-derived cells into the distal ventricle. Embryos treated with 20 μM and 50 μM VPA presented with a heart looping defect. Those subjected to 50 μM or 100 μM VPA had uninflated ventricles (Fig. 2A, b,b‘,c,c‘,d,d‘,e,e‘).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.chemosphere.2019.04.061

The embryos treated with 50 μM VPA had distal ventricles that were partially ruptured at the border of the outflow tract (Fig. 2A, c, arrow; Supplementary Video C). In 53-hpf embryos treated with 50 μM and 100 μM VPA, the edge of the small/collapsed ventricle was physically disconnected from the outflow tract resulting in a cluster of EGFP-positive cells at the arterial pole of the outflow tract (Fig. 2A, d,e, arrow; Supplementary Videos D,E). The distances between the two edges of the distal ventricle and the arterial pole were 164 μm and 266 μm in the embryos exposed to 50 μM and 100 μM VPA, respectively (Fig. 2A, d‘,e‘; Supplementary Videos D,E).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.chemosphere.2019.04.061

Interruption of myl7 mRNA expression was the consequence of the physical split of the heart from the outflow tract. VPA-associated heart dislocation was statistically quantified based on the in situ hybridization data (Fig. 2B, P < 0.01).

3.3. Zebrafish embryo with VPA-associated heart dislocation has limited viability

We investigated the relationship between VPA-associated dislocation of the ventricle from the outflow tract and embryo survival. All embryos treated with 50 μM and 100 μM VPA died by 6 dpf and 8 dpf, respectively (Fig. 3A). However, the heartbeat was robust even in embryos whose distal ventricles were dislocated from outflow tracts at 48 hpf. The heart rate of the embryos treated with 100 μM VPA was only 20% that of the control (Fig. 3B). These embryos remained viable and thus their cardiac phenotypes could be imaged in vivo.

3.4. VPA causes severe defects in endocardium development

Both myocardial and endocardial cells were reduced in the ventricles and atria in a VPA dose-dependent manner (Fig. 4). Cell counting in 3D video images revealed the most dramatic decrease of endocardial cells in the ventricles of VPA-treated embryos (Figs. 2A and 4B). The present data demonstrate that VPA causes very severe defects in endocardium and moderate defects in myocardium at the embryonic stage.

3.5. Folic acid (FA) relieves VPA-associated hypoplastic heart

The 0.2 μM FA supplement increased myl7 RNA expression in the heart chambers (Fig. 5A). myl7 was either upregulated or detected at the edge of the disconnected outflow tract in 48-hpf embryos supplemented with 0.2 μM FA (Fig. 5A, d,e‘). Whereas the incidence of unloop heart was 35% in embryos exposed to 20 μM VPA alone, it was 26% in embryos receiving 0.2 μM FA. In contrast, 0.2 μM FA actually increased the number of dislocated hearts in 50 μM VPA-treated embryos compared to that in those exposed to 50 μM VPA alone (Fig. 5B, P < 0.01). The 0.2 μM FA treatment reduced the incidence of incomplete heart looping that occurred in a few wild type embryos because of random interbatch egg quality variations (Fig. 5B, P < 0.01). During earlier embryonic development, myl7 expression was also decreased in a VPA dose-dependent manner and the 0.2 μM FA supplement increased myl7 RNA expression in the heart field and heart tube at 24 hpf and 36 hpf (Supplementary Fig. 1, P < 0.05). These results suggest that 0.2 μM FA supplementation reduces the severity of VPA-associated cardiac phenotypes.

FA effect on VPA-associated cardiac defect was visualized in 53hpf Tg(cmlc2:egfp/kdrl:mcherry) embryos using 3D light-sheet microscope (Fig. 6A). The 0.2 μM FA supplementation significantly increased the number of myocardial and endocardial cells in all embryos (Fig. 6B, P < 0.05). FA supplementation during embryo development may reduce spontaneous congenital heart defects including hypoplastic heart during chamber development.

Relative to control embryos, those treated with various VPA concentrations from 5 hpf to 24 hpf showed VPA-associated decreases in heart rate at 48 hpf (Fig. 6C). The heart rates of the embryos supplemented with FA were significantly higher than those of both controls (Fig. 6C). FA supplementation relieved both
VPA-associated and spontaneously occurring heart defects. Moreover, the cellular changes that occurred in every case were proportionately reflected in cardiac function during embryonic development.

4. Discussion

In the present study, light-sheet profiling of early cardiac development in live zebrafish embryos revealed cardiotoxicity and elucidated the mechanism of drug cardiotoxicity underlying congenital heart diseases. It provided 3D differentiation of the cellular changes in the second heart field-derived cardiac tissues within the heart and the outflow tract. Our live 3D imaging by light-sheet microscopy visualized the physical disconnection of the heart from the arterial pole of the outflow tract (a second heart field-derived structure) in 53-hpf embryos treated with 50 or 100 μM VPA. In this way, VPA cardiovascular toxicity could be assessed in zebrafish embryogenesis for the first time. Detection of this
phenotype was made possible by a combination of the advantageous transparency of the zebrafish embryo and light-sheet imaging technology. A unique feature of zebrafish embryos is that they remain viable for a limited time in the absence of a heartbeat (Stainier et al., 1996). Moreover, the present study showed for the first time that the zebrafish embryo can temporarily survive when its heart is physically dislocated from the arterial pole of the outflow tract. These traits may have furnished the time window required to image the process of abnormal cardiovascular development and to investigate the mechanisms underlying the toxic effects of various chemicals and drugs on cardiac morphogenesis at the cellular level. In contrast, cardiovascular dislocation may cause immediate death in other vertebrates.

During vertebrate embryogenesis, the earliest cardiomyocytes in first heart field progenitors differentiate bilaterally in the lateral plate mesoderm, migrate to the midline, and assemble the linear heart tube. On the other hand, second heart field progenitors remain undifferentiated in the lateral plate mesoderm and reside in the pharyngeal mesoderm where the poles of the linear heart tube attach to the embryo proper (Zhou et al., 2011; Guner-Ataman et al., 2013). Within a well-defined developmental window, second heart field progenitors differentiate into nascent cardiomyocytes that are progressively added to the poles of the heart tube (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). These processes are highly evolutionarily conserved within vertebrates. Even subtle perturbations of these critical steps may cause severe congenital cardiovascular defects. In zebrafish, successive phases of cardiomyocyte differentiation generate the embryonic myocardium. Within the first 24 hpf, the first heart field-derived cardiomyocytes form the linear heart tube comprising both the atrial and ventricular cardiomyocytes (Yelon et al., 1999; de Pater et al., 2009; Lazic and Scott, 2011; Jahangiri et al., 2016). Between 24 and 48 hpf, the second heart field progenitors differentiate into cardiomyocytes that are progressively accreted to the arterial pole of the heart tube (de Pater et al., 2009; Hami et al., 2011; Lazic and Scott, 2011; Zhou et al., 2011). When accretion is complete, the first- and second heart field-derived cardiomyocytes occupy roughly the proximal and distal halves of the ventricular myocardium, respectively.

Hypoplastic right ventricle and septal defects were reported for VPA teratogenicity in mice, rats and rarely in humans (Binkerd et al., 1988; Sonoda et al., 1993; Menegola et al., 1996; ten Berg et al., 2005; Ozkan et al., 2011). However, further investigation is necessary to understand the mechanism accounting for the relatively rare occurrence of this pathogenicity in humans. The right ventricle and aorta are formed by progression of the second heart field-derived development of the four heart chambers in the human fetus. Abnormalities in the second heart field-derived structures of zebrafish associated with fetal valproate syndrome may reflect hypoplastic right ventricle, aortic or pulmonary stenosis, and septal defects.
Our light-sheet imaging visualized the endocardium in live zebrafish embryos and revealed severe defects in endocardial development and myocardial development associated with VPA exposure. FA supplementation alleviated the heart looping defect and increased the numbers of myocardial and endocardial cells in heart chamber development and the accretion of second heart field-derived myocardial cells to the arterial pole of the outflow tract in all embryos. Therefore, FA supplementation proportionately increased the heart rate which is indicative of an improvement in functional cardiac output (Figs. 5 and 6). A cardiomyocyte cell culture scratch assay showed that VPA-associated inhibition of cardiomyocyte motility was rescued by FA supplementation in vitro (Yu et al., 2016). Arterial pole cells significantly increased at the tip of the disconnected outflow tract in the presence of FA. Therefore, FA supplementation may facilitate cardiomyocyte migration from the pharyngeal arch to the arterial pole of the heart tube between 24 and 48 hpf. In 48-hpf zebrafish embryos, endocardial cells undergo an epithelial-to-mesenchymal transition at the chamber boundaries, form cardiac cushions, and produce heart valves (Stainier, 2001). Light-sheet imaging clearly visualized both VPA-associated defects and FA-induced relief in endocardium formation at the cellular level. It also enabled the quantification of the endocardial cells in each chamber via 3D video images (Fig. 6A). The results substantiated the putative role of FA supplementation against the spontaneous occurrence of hypoplastic endocardium. On the other hand, FA supplementation actually increased the frequency of VPA-derived physical heart dislocation (Fig. 5B). Therefore, cell-cell adhesion at the junction may not be strong enough to withstand pulling at 36–48 hpf when FA supplementation facilitates heart looping and the heart primordia complete rightward looping.

Light-sheet imaging is rapidly gaining traction as the method of choice for the optical investigation of biological activity. It creates both opportunities and challenges in the elucidation of the events occurring on the biological canvas. Recent progress in confocal microscopy allows ultrafine imaging of organelles and smaller intracellular structures at high resolution. In contrast, light-sheet microscopy does not yet have this capacity. However, the present study demonstrates that light-sheet imaging is a powerful assay in certain ways superior to confocal microscopy, two-photon microscopy and their derivatives in terms of scale and speed. Light-sheet microscopy can be used in the routine visualization and quantification of the cellular changes occurring in the heart and surrounding tissues of zebrafish embryos. It may also differentiate...
heart field-derived cardiotoxicity in humans and animals.

5. Conclusions

Live 3D imaging of zebrafish embryo by light-sheet technology revealed a novel valproate cardiotoxicity phenotype consisting of physical dislocation of the ventricle from the outflow tract and incomplete formation of the distal ventricle. Physical disconnection of the heart has not been previously reported for any other model system. The present approach combining the zebrafish embryo with light-sheet imaging under experimental conditions may overcome the limitations of conventional imaging technology. Our 3D light-sheet imaging is sensitive enough to quantify the changes in the myocardium and the endocardium at the cellular level while covering both the heart and its surrounding structures during zebrafish heart development. The 3D light-sheet assay used in the present study to assess valproate-associated cardiotoxicity and folic acid relief highlighted the utility of state-of-the-art 3D light-sheet microscopy combined with the zebrafish model as a valuable toxicological assay for chemical cardiotoxicity at the cellular level. This approach may elucidate the mechanisms underlying congenital cardiovascular defects in humans and animals.
Authors contribution

Yun Kee and Byung Joon Hwang conceived and designed experiments and analyzed data. Yun Kee, Byung Joon Hwang, and Seoyoung Ki wrote the paper. Seoyoung Ki, Seung-Hae Kwon, and Juneyong Eum performed experiments and analyzed data, and Ahmed A. Raslan, helped analyze data. Seung-Hae Kwon and Kil-Nam Kim contributed analysis tools and helped acquisition of data.

Ethical statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Declarations of interest

None.

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Appendix A. Supplementary data

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References


