Targeted Laser Ablation of the Zebrafish Larval Heart Induces Models of Heart Block, Valvular Regurgitation, and Outflow Tract Obstruction

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Abstract

Mammalian models of cardiac disease have provided unique and important insights into human disease but have become increasingly challenging to produce. The zebrafish could provide inexpensive high-throughput models of cardiac injury and repair. We used a highly targeted laser, synchronized to fire at specific phases of the cardiac cycle, to induce regional injury to the ventricle, atrioventricular (AV) cushion, and bulbus arteriosus (BA). We assessed the impact of laser injury on hearts of zebrafish early larvae at 72 h postfertilization, to different regions, recording the effects on ejection fraction (EF), heart rate (HR), and blood flow at 2 and 24 h postinjury (hpi). Laser injury to the apex, midzone, and outflow regions of the ventricle resulted in reductions of the ventricle EF at 2 hpi with full recovery of function by 24 hpi. Laser injury to the ventricle, close to the AV cushion, was more likely to cause bradycardia and atrial–ventricular dysfunction, suggestive of an electrical conduction block. At 2 hpi, direct injury to the AV cushion resulted in marked regurgitation of blood from the ventricle to the atrium. Laser injury to the BA caused temporary outflow tract obstruction with cessation of ventricle contraction and circulation. Despite such damage, 80% of embryos showed complete recovery of the HR and function within 24 h of laser injury. Precision laser injury to key structures in the zebrafish developing heart provides a range of potentially useful models of hemodynamic overload, injury, and repair.

Introduction

MODELS OF CARDIAC DISEASE and cardiac impairment in mammals have provided unique and important insights into human disease. However, increasingly, the ethical and practical challenges of inducing cardiac injury in small mammals have encouraged researchers to seek alternative relevant models of human disease. Development of the zebrafish (*Danio rerio*) heart has been extensively studied and has yielded important information on key molecular pathways during early development (for review, see Staudt and Stainier¹). In evolutionary terms, the zebrafish is closer to humans than *Caenorhabditis* and *Drosophila*. Overall, 70% of human genes have a zebrafish orthologue, whereas 82% of genes known to be responsible for specific human diseases have been determined to have orthologues in zebrafish.² Nevertheless, there remains a lack of models of cardiac injury similar to those used in small rodents, such as aortic banding, myocardial infarction, and valvular regurgitation.

The zebrafish could conceivably provide an inexpensive, high-throughput, and humane set of models of cardiac impairment.³ As in mammals, the zebrafish developing heart undergoes a series of complex morphogenetic and differentiation processes to form a mature cardiac structure.^{4–6} Perturbations in these processes can lead to structural abnormalities, which are challenging to image and hence characterize⁷ and which are associated with embryonic lethality.

The zebrafish embryo/larva (see chapter 5 in Nüsslein-Volhard and Dahm⁸ for developmental stage nomenclature) circumvents these issues due to its high degree of transparency making the developing heart easily visualized by standard or fluorescent microscopy and by virtue of the fact that the larva can survive for up to 120 hpf without a beating heart and without a heme-based circulation.⁹ Such unique larval survival permits the assessment of severe cardiovascular abnormalities that would usually be embryonic lethal. These properties, together with the high number of offspring and external development, make the zebrafish embryo/larva a

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versatile model for cardiovascular disease models and mechanisms.

Techniques allowing highly targeted injury of specific cardiac regions and anatomical structures could provide novel models of cardiac impairment, in which to test the impact of pharmacological and genetic interventions. Laser ablation techniques have been previously used in the zebrafish embryo to induce thrombosis,¹⁰ ischemia,¹¹ kidney injury,¹² brain injury,¹³ and heart injury.^{14,15} Similar to the adult zebrafish heart,^{16,17} the embryonic heart shows a rapid regenerative and functional recovery following injury most likely due to powerful proliferative mechanisms already activated during this key developmental time period.¹⁵

In this study, we assessed the hemodynamic and functional effects of laser injury to specific cardiac regions, including the ventricular apex, mid, and outflow tract (OFT) zones, and we visually assessed the hemodynamic effects of laser injury to the atrioventricular (AV) cushions and the bulbus arteriosus (BA).

Methods

Ethical approval

All experiments were approved by the local ethics committee and conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 in an approved establishment.

Zebrafish maintenance

Zebrafish husbandry, embryo collection, and maintenance were performed according to the accepted standard operating procedures.⁸ The cardiac myosin light chain 2:GFP transgenic line (tg[cm1c2:EGFP])¹⁸ was used for all experiments, unless stated; embryos/larvae were maintained at 28.5°C on a 14-h light/10-h dark cycle and staged according to Kimmel.¹⁹ Embryos were kept in egg water until dechorionated and larvae were kept in the embryo medium.²⁰ All experimental procedures were performed at room temperature (RT, 23°C).

Heart laser injury

Larvae (72 hpf) were anesthetized using Tricaine 20 μ M (Sigma-Aldrich, Dorset, England) and were placed on their side on a plain glass microscope slide in a minimal amount of water. Live images were captured by a digital video camera mounted on a microscope and displayed on a monitor. The Infrared Laser Ablator XYClone (Hamilton-Thorne, Beverly, MA) was directed at a specified region of the heart using a built-in collimated red light beam target that indicates the position of the laser beam in the field of view (Fig. 1 and Supplementary Movie SM1 Supplementary Data are available online at www.liebertpub.com/zeb). The average energy delivered per pulse was 0.9 mJ over a duration of 3 ms (300 mW). The laser system was linked to a personal computer with custom-built software that permits synchronization between delivery of the laser pulse and the phase of the cardiac cycle¹⁵; the optical gating software is described in detail elsewhere.²¹ This approach allowed us to create precise injury to three different regions of the ventricle: basal (B), mid (M), and apex (A), and to the AV cushions and the ventricle OFT near the BA.

FIG. 1. Targeted laser injury to the zebrafish embryonic heart. Embryos at 72 hpf (**A**) were placed under a compound microscope equipped with a video camera and an objective 20 $\times$ linked to the Laser Ablator (XYClone, Hamilton Thorne). The laser was targeted at specific regions of the ventricle and at specific cardiac structures (**B**), including the atrioventricular cushion (AV), bulbus arteriosus (BA), and base (B), mid (M), and apex (A) regions of the ventricle. Color images available online at www.liebertpub.com/zeb

After injury, each larva was immediately placed in the embryo medium to recover. Control larvae, kept in identical conditions and manipulated in the same way, received laser injury to the distal tail fin.

Assessment of cardiac function and hemodynamics

Images of the beating heart were captured by a video camera (IonOptix CCD100 MyoCamtm, Milton, MA) mounted on a microscope (Zeiss, Axioscope II MOT Plus, Thornwood, NY) linked to a PC. The ejection fraction (EF) and heart rate (HR) were determined using image analysis software (ImageJ; NIH, Bethesda, MD). The EF was estimated using a simple area subtraction method, which subtracts ventricular systolic area from diastolic area and expresses this as a percentage of diastolic area.²² Contraction rates of the atrium and the ventricle were assessed separately using the video images for a period of at least 10 s and expressed as beats per minute (bpm) for each heart chamber. Blood flow in the zebrafish larva was analyzed by visual observation of the red cell movement in the caudal vein and reported as normal or reduced/absent.

Statistical analyses

Experiments were performed in triplicate on an average of 20–30 larvae per experiment, unless otherwise stated. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5. One-way or two-way repeated measures ANOVA followed by the Bonferroni *post hoc* test was used to compare means

FIG. 2. Effects of laser injury to different heart ventricle regions (apex, mid, and basal zones). Ejection fraction (A) and heart rate (B) following a single laser pulse to the basal, mid, or apex regions of the ventricle. Data presented are mean $\pm$ SEM. ** $p < 0.01$, ANOVA test followed by the Bonferroni *post hoc* test (Supplementary Movie SM2).

within and between groups. p Values < 0.05 were considered significant.

Results

Zebrafish larval hearts were injured at 72 hpf, and the impact of laser injury was assessed at 2 and 24 h postinjury (hpi). Laser injury targeted at the ventricle base, mid, and apex zones using a single laser pulse (Fig. 1) caused a similar reduction in the EF at 2 hpi of $14\% \pm 1\%$, $13\% \pm 1.1\%$, and $13\% \pm 0.8\%$ for the respective regions (Fig. 2A), following which there was a full functional recovery by 24 hpi in all the three groups. At 2 hpi, the ventricle contraction rate (VR) was significantly reduced in all the injured groups (Fig. 2B). This was accompanied by the absence of forward blood flow in most (70–75%) of the embryos injured (Table 1). At 24 hpi, the EF and VR had returned to normal in the majority (90%) of embryos from each group (Fig. 2 and Table 1).

Laser injury to the AV cushion resulted in a reduced EF (Fig. 3A) and diminished forward blood flow (Table 1). Injury to this region frequently resulted in features of heart block observed immediately after the injury (Supplementary Movie SM1) and at 2 hpi as a marked reduction in the VR (Fig. 3B, C) with preserved atrial contraction rate (ventricle:atrium rate ratio 1 ± 0.06 vs. 0.6 ± 0.1 , $p \leq 0.01$). Injury to the AV cushion also resulted in marked regurgitation of blood cells from the ventricle into the atrium with the evidence of reverse pulsatile flow extending into the caudal vein consistent with valvular regurgitation in 78% of larvae (Table 1).

TABLE 1. PROPORTION (%) OF LARVAE DISPLAYING ABNORMAL BLOOD FLOW PATTERNS IN THE HEART 2 AND 24 HOURS POST LASER INJURY (HPI) TO DIFFERENT REGIONS OF THE HEART [ATRIOVENTRICULAR (AV) CUSHION, BASE, MIDDLE (MID) AND APEX OF VENTRICLE (V)] ($N = 20$ –30 LARVAE PER VENTRICLE REGION, $N = 3$ EXPERIMENTS)

	Injury type					
	Control no injury	AV cushion	V-Base	V-Mid	V-Apex	BA
Ventriculoatrial regurgitation (%)						
2 hpi	0	78	12	10	15	85
24 hpi	0	18	5	0	0	25
No forward blood flow (%)						
2 hpi	0	80	70	75	70	75
24 hpi	0	22	5	10	5	12

The VR, AV synchrony, and ventricle EF returned to control levels by 24 hpi, although 18% and 22% of larvae continued to show evidence of ventriculo-atrial (VA) regurgitation and reduced anterograde blood flow, respectively.

Laser injury to the BA resulted in rapid thrombotic occlusion of the OFT, followed by an accumulation of erythrocytes within the ventricle cavity and cessation of circulation to the gills and tail (Supplementary Movie SM2). In some cases, the flow through the BA resumed after 15–20 min followed by a period with reduced forward flow. At 2 hpi, these hearts continued to show a significant reduction in the HR and EF (Fig. 4), with 85% and 75% of larvae showing VA regurgitation and reduced anterograde blood flow, respectively (Table 1). At 24 hpi, the HR and EF showed values similar to controls, with VA regurgitation and reduced blood flow evident in 25% and 12% of embryos, respectively.

Discussion

This study has explored the use of a laser to create injury of specific cardiac structures in the zebrafish larval heart at 72 hpf. The unique advantage of the system applied in these experiments is its association with software that permits synchronization of laser firing with heart movement, allowing highly targeted and localized injury. For example, the valve structures are crucial for maintaining unidirectional blood flow in the heart, and AV canal defects represent the third most common heart defects in humans.²³ A single laser pulse to the AV cushions resulted in immediate bradycardia, lasting for a few seconds, followed by a significant reduction in the ventricular contraction rate (VR) that gradually recovered over the following 2 h returning to normal by 24 hpi. Injury to the AV cushion resulted in a marked reduction in the VR, while the atrial contraction rate remained at or close to normal, suggesting an AV conduction block. Laser injury to this region of the ventricle clearly caused temporary damage to the electrical conducting system in the larval heart. Such a system has been described previously,^{24,25} although a conducting system has not been clearly defined anatomically.^{26,27} More recently, others have suggested that the adult zebrafish does not have a specialized conduction system, and electrical activation of the ventricle occurs as a wave beginning in the dorsal-basal region and sweeping toward the apex.²⁸ Our study has clearly shown that targeted injury to the AV cushion region can create a degree of electrical block, suggesting that there is indeed cardiac tissue responsible for maintaining AV contractile synchrony in the developing

FIG. 3. Effects of laser injury to the AV cushion. Zebrafish embryos at 72 hpf received a single laser pulse at the AV cushion region. Diagrams show the ejection fraction (A), ventricle heart rate (B), and the AV rate ratio (C), analyzed before laser (baseline) and at 2 and 24 h postlaser injury. Data presented are mean $\pm$ SEM. ** $p < 0.01$, ANOVA test followed by the Bonferroni *post hoc* test (Supplementary Movie SM1).

zebrafish heart.²⁷ The question of whether there is specialized conducting tissue remains unanswered.

Injury to the AV cushion also caused marked regurgitation of blood from the ventricle into the atrium with evidence of pulsatile flow extending backward into the caudal vein. This VA regurgitation mimics mitral/tricuspid valve regurgitation commonly observed in humans.²⁹ This may result from either incompetence of the fine leaflets that are forming at this time or could be due to distortion of the AV cushion architecture resulting in incompetence in the primitive valve-ring structure. Interestingly, this hemodynamic abnormality had resolved in over 80% of the embryos injured in this way by 24 h after laser injury, suggesting that the rapid proliferation of cardiomyocytes at this time can readily compensate for the damage. Further studies would be required to assess whether more severe laser-induced injury possibly at a later time point could be used to create a model of chronic volume overload of the ventricle extending into adulthood using this technique.

Cardiomyocyte cell lineage tracing has indicated that at least three populations of cells can contribute to the mouse and zebrafish cardiac morphogenesis.^{30,31} Cell lineage has also shown the zebrafish ventricle to be heterogeneous from inner to outer myocardium.³² In addition, the different cardiomyocyte architecture is known to affect electrical impulse propagation across the myocardium.³³ Recently, outer curvature and inner curvature cardiomyocytes in wild-type

zebrafish ventricles have been reported to acquire distinct cell shapes during chamber formation, with their final shape depending on contractile function.⁴ We hypothesized that the zebrafish heart ventricle would respond differently, particularly in terms of regenerative potential, when injured in different regions. However, laser injury to each of three distinct ventricle regions resulted in similar drops in the EF and ventricular rate at 2 hpi with recovery to control values by 24 hpi in all three groups.

Laser technology has been previously used to induce occlusion of the dorsal aorta to induce arteriogenesis and collateral blood vessel development in the zebrafish embryo/larva.¹¹ In this study, we have used our laser model also to induce complete or partial ventricle outflow obstruction in the beating zebrafish embryo heart. The BA, also called cardiac OFT, is a short connection situated between the contracting muscular heart chamber and the vascular network. This region has been widely studied by others interested in congenital heart defects.³⁴ One or two laser pulses to the BA caused temporary or permanent obstruction to cardiac outflow. Following outflow occlusion, the atrium continued to contract vigorously while erythrocytes accumulated rapidly in the ventricle. The ventricle appeared to show signs of volume and possibly pressure overload with marked thickening of the ventricle wall as it continued to contract against an obstructed OFT. This also resulted in marked AV regurgitation in many hearts. This

FIG. 4. Effects of laser injury to the BA. Ejection fraction (A) and heart (ventricle) rate (B) at 2 and 24 h following laser injury. Zebrafish embryos at 72 hpf received a single laser pulse at the BA close to the outflow tract. Data were presented as mean $\pm$ SEM. ** $p < 0.01$, ANOVA test followed by the Bonferroni *post hoc* test (Supplementary Movie SM2).

technique requires further studies to explore whether a longer term pressure overload model could be created in the adult due to chronic OFT obstruction induced by injury to the larval heart. Such a model could provide a relatively inexpensive model of cardiac pressure overload.

Laser-induced injury of different cardiac regions and structures in the zebrafish larva demonstrates the potential of the zebrafish to be used for multiple models of cardiac disease that would complement those used in mammals.

There are clearly significant differences between the zebrafish and mammals, including the complexity of the heart, which raises some questions about the relevance of these models compared with more traditional mammalian models of cardiac disease. However, the zebrafish larva represents a unique model system, including its remarkable rapid regeneration–repair mechanisms. As a model system, and with these unique characteristics, it can be combined with features developed in mammalian model systems, such as valvular regurgitation, to provide entirely novel experimental approaches. Combining these approaches with high-throughput drug screening programs could, for example, be used to efficiently explore novel therapeutic candidates before advancing to more expensive mammalian models. Compared to the adult, the zebrafish larva not only offers the advantage to study hundreds of animals over a short time period but also allows the possibility to study severe developmental cardiac defects, which would otherwise be lethal in mammalian models. Therefore, studies in the zebrafish larva have the potential to provide enormous insights, including targeted injury to the heart. The work presented here therefore represents an important step forward by combining the advantages of the zebrafish larva with traditional models of mammalian cardiac disease. Further work is clearly needed to establish the utility of these models for ongoing cardiovascular research.

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Disclosure Statement

No competing financial interests exist.

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