Original Research

Femtosecond laser photodisruption of vitelline vessels of avian embryos as a technique to study embryonic vascular remodeling

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Abstract

During cardiogenesis, congenital heart defects (CHDs), generally start as local tissue abnormalities without underlying genetic causes, suggesting abnormal hemodynamics may be an important source. Due to the scarcity of experimental techniques that permits the formation of minimally-invasive and well-controlled cardiac perturbations, experimental investigation of embryonic development of CHD via *in-vivo* models is difficult. In this study, in order to investigate the relationship between abnormal mechanical signaling and embryonic CHD development, a previously developed laser-based technique was adopted to alter chicken embryonic cardiovascular development. The technique incorporates two-photon fluorescence microscopy to visualize deep tissue while femtosecond-pulsed laser photodisruption is used to ablate targeted tissue. Vitelline vessel remodeling under abnormal hemodynamics was the prime concern of the study. In order to alter the hemodynamics, blood flowing inside 50–300 μm diameter Hamburger–Hamilton 24 embryonic vessels was selectively ablated. Red blood cells in the blood and endothelial cells of the vessel walls were damaged as a result of ablation. Cellular injuries led to micro-occlusions in the vessels. Several micro-occlusions formed stable clots, resulting in a complete cessation of blood flow in the targeted vessels. By measuring blood velocities in the surrounding vessels via line scanning technique, the subsequent redistribution of blood flow in the immediate upstream and downstream vessels was revealed. The network was analyzed after 24 h, and it was found to be degraded. Degradation of the entire network can be attributed to the abnormalities in hemodynamics within the vessels. For studying embryonic development of heart defects under disturbed flow conditions, the present study can be extended to clot a blood vessel inside the embryo or a vitelline vessel in the vicinity of the heart. These results demonstrate that, laser-based noninvasive tools should be considered as powerful techniques to analyze hemodynamic signals encountered in embryonic development of CHD.

Keywords: Chick embryo, hemodynamics, vascular remodeling, femtosecond lasers, two-photon microscopy, congenital heart defects

***Experimental Biology and Medicine* 2014; 239: 1644–1652. DOI: 10.1177/1535370214546272**

Introduction

Cardiovascular system is the first system that starts to function in an embryo. The development of the blood vessels and the heart continues under constantly changing blood flow environment.[1] With the recent developments in the *in-vivo* high resolution imaging modalities[2–4] and computational modeling techniques,[5–8] blood flow characteristics inside developing hearts were studied in detail and specific blood flow patterns were identified. Deviations from normal hemodynamic conditions, and therefore, disrupted blood flow patterns affect cardiovascular development as observed in numerous clinical studies.[9] Abnormal hemodynamic forces are thus accepted as important sources of congenital heart defects (CHDs). In controlled experiments, animal embryos were used to study the development of the cardiovascular system under abnormal blood flows. In these studies, blood flow is altered via microsurgery to reproduce the defective hemodynamics which is encountered in clinical observations. Two widely used model systems are zebrafish and chicken embryos. In order to constrict blood flow through the heart, Hove *et al*. inserted microbeads into the inflow or outflow tracts of zebrafish embryos and demonstrated that flow constrictions resulted in the formation of an additional third chamber and abnormal valve formation.[10] Ligating the left atria of chicken embryos (a technique known as left atrial ligation, LAL) was shown to change intracardiac blood flow patterns,[11] thus resulting in the remodeling of ventricular myoarchitecture,[12] and aortic arches.[13] Constricting the blood flow in the

outflow tract via conotruncal banding was shown to increase the pressure load in the heart which then led to valvular[14,15] and conduction system anomalies.[15] Likewise, Hogers *et al*. showed that ligating any of the three major vitelline veins of chicken embryos causes alterations in intracardiac blood flow patterns[16] which result in ventricular, valve, and pharyngeal arch anomalies.[17] These findings suggest that altered hemodynamic forces may be an important source for CHDs.

LAL and other CHD models are important in that they identify the deviations from normal hemodynamics during heart development as the cause of subsequent heart defects. However, they suffer from the disadvantage that the induced tissue malformations can be so severe and the result could be far from that of clinical occurrence. Therefore, better controlled and less invasive techniques to generate animal CHD models are necessary to investigate the relationship between the hemodynamics and tissue growth in cardiovascular development.

Recent developments in nonlinear optics made it possible to induce micrometer-sized defects beneath 1–2 mm tissue surfaces while keeping the surrounding regions intact.[18] In this method, high-energy femtosecond-pulsed lasers are tightly focused onto the tissue to be ablated. Energy is absorbed in a nonlinear fashion at the focal point of the focusing lens, constricting the damage into a region of μm^3 order. Nishimura *et al*. combined this ablation technique with a two-photon (2 P) microscopy system to simultaneously visualize and disrupt small blood vessels in rat brains.[19,20] To study the development of valvular heart defects, the author in his previous work[21] used this system to induce controlled micrometer-sized tissue defects into the primitive atrioventricular (AV) valves of chicken embryos. In the current study, the same system is utilized to study the vascular remodeling of embryonic blood vessels under altered blood flow environments. Blood flowing inside vitelline vessels was irradiated with a femtosecond laser to induce clot formation inside these vessels (vitelline vessels are the vessels spreading on the yolk that are critical for the nutrition transport to the avian embryos). Flow velocity measurements in the vessels through line scanning revealed that blood flow constriction in a vessel causes rapid redistribution of blood flow in the neighboring vessels. Redistribution of the flow resulted in local vascular remodeling in 24 h. This technique can be further used to clot a blood vessel inside the embryo or a vitelline vessel close to the heart for the purpose of studying the embryonic heart development under abnormal hemodynamic conditions.

Methods

Chicken embryo culture

Fertilized chicken eggs were kept in an egg incubator at 37.5°C (Avery Incubators, Hugo, CO) till Hamburger–Hamilton (HH) stage 19–20 (embryonic day 3). Subsequently, eggs were transferred onto the culture systems as described in the reference.[22] In this *ex-ovo* culturing technique, yolks with embryos on top are transferred over a thin plastic membrane (Saran Wrap) stretched on top of a plastic cup. Plastic cup is partially filled with sterile water as seen in Figure 1a. Before starting the experiments, embryos were incubated until stage HH24 (embryonic day 4). Vitelline vessels of an HH24 embryo can be seen in Figure 1b.

Embryo preparation

To image the embryonic vitelline vessels with the 2 P microscope, approximately 1 µL Texas red-dextran (2 MDa, 5% wt/vol in EPBS, Sigma-Aldrich) was injected into the circulating blood. One milliliter ID capillary tubes were pulled with a capillary puller and shaped in the form of a sharp tip needle with the use of a microforge. Tip diameter is typically 15 µm. Dye was slowly injected into a small vitelline vein (approximately 100 µm in diameter) over 1 min (Supplemental Movie 1), using an injection manipulator (M3301L, WPI, Sarasota, FL).

2 P microscopy

In this study, a custom-built 2 P excited fluorescence microscopy system was utilized[19,21] (Figure 2). Briefly, in this

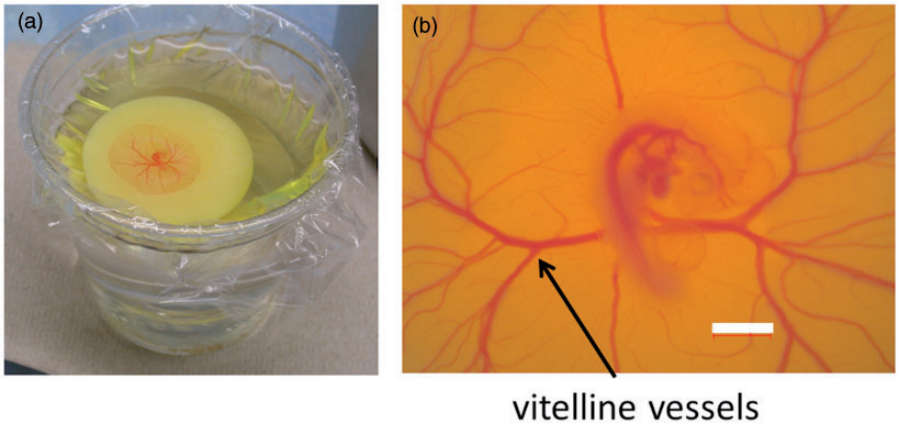

Figure 1 *Ex-ovo* culture system. a. Eggs are cracked at HH19-20 (embryonic day 3) onto the culture system. b. Vitelline vessels of an HH24 (embryonic day 4) embryo. These vessels spread onto the yolk in a planar fashion enabling optical access. Scale bar is 2 mm.

system, laser pulses with femtosecond duration (Model Mira-HP, Coherent, IMRA America) are focused onto the sample. Galvanometric mirrors are then used to scan the sample to generate 2P florescence images. The excited fluorescence light is projected onto photomultiplier tubes allowing only the fluorescence to reach the detector. A 1.0 numerical aperture 20X water-immersion objective (Zeiss) was used for imaging at a rate of 10 frames per second. The system enabled to visualize and ablate structures up to a depth of approximately 1.8 mm. For the embryonic stage studied such an ablation depth was found to be sufficient. During the experiments, cultured embryos were kept at 37.5°C by placing the culture system into a custom-built heater (Figure 2b).

Femtosecond-pulsed laser photodisruption (FPLP)

An amplified laser system (Model Legend, Coherent, Santa Clara, CA) generates high-energy, 50-femtosecond laser pulses at a frequency of 1-kHz. Using a polarizing beam splitter, this ablation laser beam is combined with the 2P imaging beam and is focused onto the 2P imaging field (Figure 2). The pulse energy for the ablation beam is adjusted with neutral density filters and the pulse rate is adjusted with a mechanical shutter having a 2-ms minimum opening time (Uniblitz, Rochester, NY). In the current study, vitelline vessels of chicken embryos were irradiated. These vessels spread over the yolk in a planar fashion and are critical for the nutrition transport to the embryo throughout the embryonic development. At early stages of chicken embryonic development (till HH27, embryonic day 6), these vessels are closer to the top portion of the yolk and are, therefore, optically easily accessible by the imaging and photodisruption systems used. The blood flow in the irradiated vessel was blocked by producing a stable clot inside the vessel. As the vessels are irradiated by the ablation laser, the endothelial cells lining the vessel walls as well as the circulating red blood cells (RBCs) in the blood were damaged. Cellular injuries resulted in a local micro-occlusion. Several micro-occlusions through the vessel were created over time in order to form a stable clot that constricts the blood flow. The mechanical shutter was kept open for about 10 s to create a single micro-occlusion.

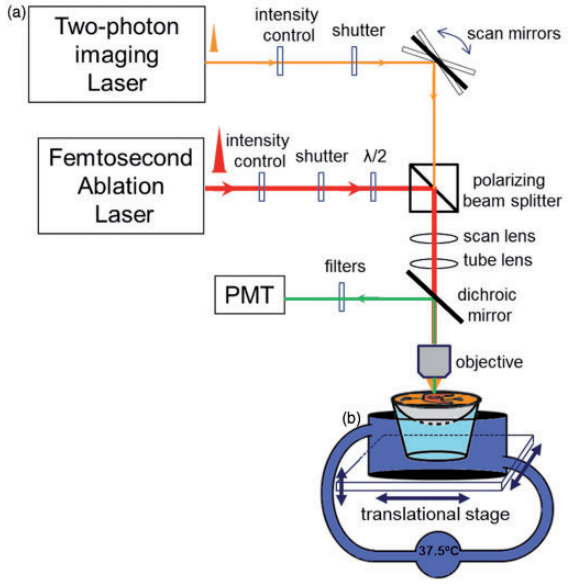

Figure 2 Experimental setup. a. Two-photon microscope modified for delivery of amplified femtosecond pulses for targeted ablation. b. Custom built water circulation heater to maintain environmental conditions during experiments. PMT, photomultiplier tube.

Blood velocity measurements via line scanning

To measure blood flow velocities in the vessels, 2P microscopy-based line scanning approach was used.[23] In this technique, longitudinal line scans at the center of the imaged vessels captures the motion of non-fluorescent RBCs parallel to the flow direction (see Supplemental Movie 2 for RBC motions). Successive scans are taken and each one is displayed below the previous one, forming a space–time image with time increasing from top to bottom. In the images, dark streaks are formed by the motion of the non-fluorescent RBCs (Supplemental Movie 3). Velocity of RBC, which is also velocity of the blood flow, is calculated from the slopes of these dark lines. Here, the slope of the lines is inversely proportional to the RBC velocity (Figure 3). In this study, for each imaged vessel, scans were taken at a rate of 1.7 kHz for 50 s. Slopes were then determined using an algorithm developed in house.[24]

Results

Visualization of vitelline vessel network via 2P microscopy

Injection of the Texas red-dextran enabled the visualization of the whole vitelline vessel network for about 2 h (Figure 4). For HH24 embryos as seen in the figure, vitelline vessels spread over the yolk in a planar fashion enabling optical access by the imaging system.

Targeted photodisruption of the flowing blood inside vitelline vessels caused micro-occlusions

When vitelline vessels were imaged via the 2P microscope, fluorescing blood plasma looked bright, and non-fluorescing RBCs looked dark (Figure 3a). Positioning FPLP laser near the center of the vessel caused the disruption of circulating RBCs and positioning near the vessel wall caused the disruption of endothelial cells lining the vessel walls (Figure 5a, Supplemental Movie 4). Disruption of RBCs and endothelial cells for approximately 10 s resulted in the accumulation of cellular debris inside the vessel. Cellular debris impairs the blood flow and cause accumulation of flowing RBCs, thus forming a local micro-occlusion. Formation of many micro-occlusions in the vessel eventually blocks blood flow completely (Figure 5b).

Determination of laser pulse energy to form a stable clot

To determine the energy levels of the irradiation that are sufficient to induce micro-occlusions in vitelline vessels of different diameters, several experiments were carried out. For this purpose, first, a vitelline vessel of approximately

200 μm in diameter was irradiated via the FPLP laser. Laser energies below approximately 400 nJ per pulse were seen not to cause any damage to RBCs or endothelium. Laser energies of the order of approximately 400–600 nJ per pulse resulted in insignificant cellular injuries. These injuries, however, did not lead to the formation of any micro-occlusions. Applying laser energies of approximately 600–800 nJ per pulse for about 10 s, on the other hand, resulted in the disruption of the RBCs as well as the endothelium. Accumulation of this damage over time resulted in the formation of micro-occlusions within the vessel. Pulses were delivered to several spots within the selected vessel to create multiple micro-occlusions that combined to form a single large stable occlusion. The vessel was successfully occluded without inducing extravasation of blood. In the occluded vessel, blood flow was completely blocked. Total clotting procedure for the above mentioned vessel took about 5 min. Laser energies of approximately 850 nJ per pulse and above resulted in the disruption of the vessel wall and caused blood leakage from the vessel (Figure 6a). To determine the clotting energies for vessels with different diameters, the same procedure was repeated. Clotting energy was found to increase linearly with the diameter of the vessel (Figure 6b).

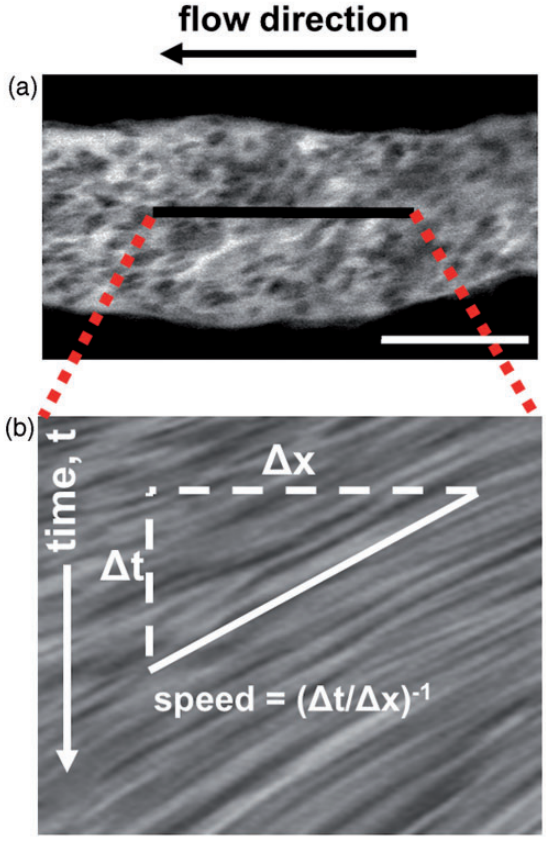

Figure 3 Line scan technique to calculate blood velocity in embryonic vessels. a. Intravenous injection of the fluorescent dye is used to distinguish between red blood cells (RBCs) seen as dark circular disks and blood plasma seen as bright flowing fluid. b. The dark streaks running from upper right to lower left formed by the motion of the non-fluorescent RBC are used to calculate blood speed (inverse of the slope). Scale bar is 2 mm. (A color version of this figure is available in the online journal.)

Clotting a vitelline vessel caused rapid redistribution of blood flows in the neighboring vessels

To observe how clotting of a vitelline vessel affects blood flow rates in the neighboring vessels, a vitelline vessel of about 200 μm diameter was clotted and the immediate vicinity was imaged. Figure 7a shows the vessel network in the field of interest. In the figure, vessel E is the clotted vessel, vessels A and B are the upstream (closer to the heart) vessels, and vessels C and D are the downstream (further away from the heart) vessels. To measure local blood velocities before ablation, each vessel in the network, labeled A through E, was first line scanned. Once vessel E is clotted, the blood velocities in each vessel were measured similarly. Occlusion of blood flow in vessel E resulted in an immediate dramatic increase of the blood velocity in the upstream vessels A and B, and cessation of blood flow in the downstream vessels C and D (Figure 7b). Representative line scan images for each vessel can be seen in Figure 7c. In order to check the accuracy of velocity measurements and reveal flow redistribution due to the insult, flow rates in the vessels before and after the occlusion were calculated using the following formula:

$$\upsilon = V \times (\pi D^2)/4$$

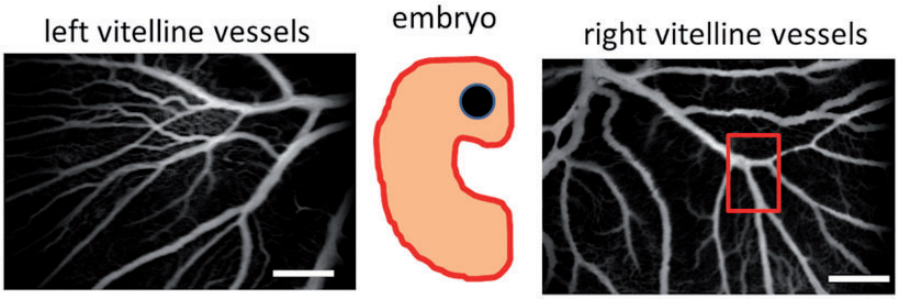

Figure 4 Two-photon microscopy imaging of vitelline vessels of an HH24 chick embryo after intravenous injection of the fluorescent dye. Red box on the right image shows the targeted vitelline vessel network shown in Figure 7. Scale bar is 2 mm. (A color version of this figure is available in the online journal.)

where
υ is the flow rate, mm^3/s
V is the average velocity in the vessel, mm/s
D is the average diameter of the vessel, mm

Flow rate values are tabulated in Table 1. Before the occlusion, total of flow rates in vessels C and D matches the flow rate in the upstream of these vessels, vessel E (24.5 *vs.* $26.7 \times 10^{-3}\,mm^3/s$). Similarly, total of flow rates in vessels E and A matches the flow rate in the upstream of these vessels, vessel B (32.1 *vs.* $29.6 \times 10^{-3}\,mm^3/s$). These results show that, velocity measurements are accurate. Minor discrepancies here are most likely due to using average flow velocities measured at longitudinal sections of the vessels instead of using velocities measured at specific cross sections. Flow rate calculations after the occlusion indicate that flow rates in the upstream vessels of the clotted vessel E increased significantly: flow rate in vessel A is 22.7×10^{-3} mm^3/s after the occlusion (*vs.* 5.4×10^{-3} mm^3/s before the occlusion) and flow rate in vessel B is 43.4×10^{-3} mm^3/s after the occlusion (*vs.* 29.6×10^{-3} mm^3/s before the occlusion).

Flow redistribution led to vascular remodeling

Next, to discover the effect of blood flow redistribution over the vascular structure, 2 P images of the targeted vessel and brightfield images of the network are captured and the results are given in Figure 8. Figure 8a and b compares normal and targeted vessel structures. While RBCs can

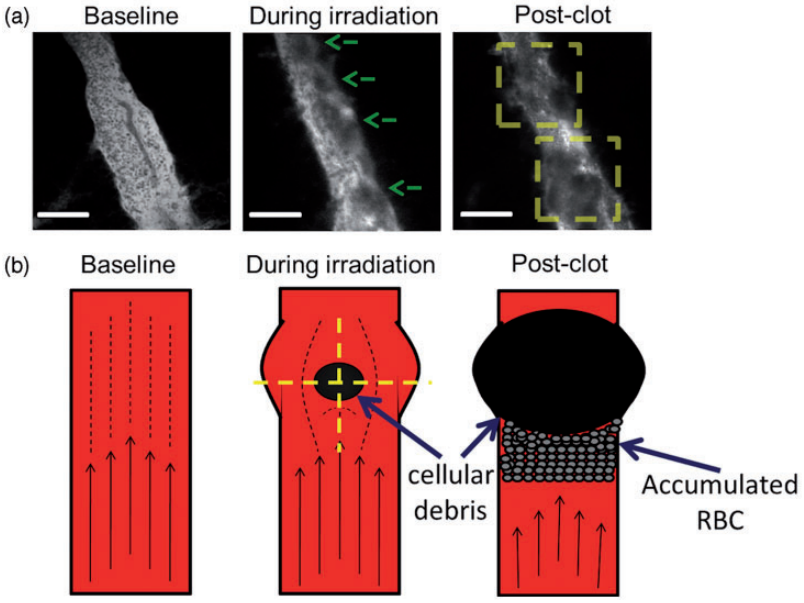

Figure 5 Photothrombotic clotting procedure in the embryonic chick vitelline vessels. a. Positioning FPLP laser near the center of the vessel caused the disruption of circulating RBCs and positioning near the vessel wall caused the disruption of endothelial cells, leading to formation of micro-occlusions (shown with green arrows in the middle image). Formation of many micro-occlusions led to a stable clot (shown with yellow boxes in the right image). Scale bar is 200 μm. b. Schematic representation of the clotting procedure. Here, arrows represent the blood flow and crosshairs in the middle image represent the target point of the FPLP laser. As seen in the right image, accumulation of RBCs contribute to clot formation

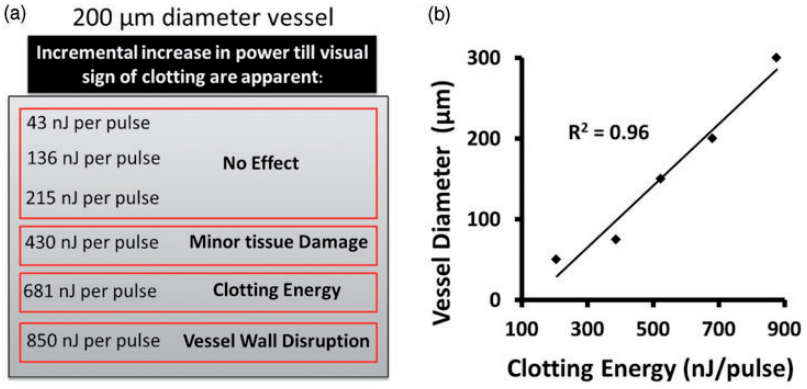

Figure 6 Determination of FPLP laser energy to form a stable clot. a. FPLP laser was targeted to a ~200 μm diameter vessel at varying energies to find the clotting energy for this vessel. b. Clotting procedure was repeated for vessels with different diameters to find out the correlation of the clotting energy with the vessel diameter. (A color version of this figure is available in the online journal.)

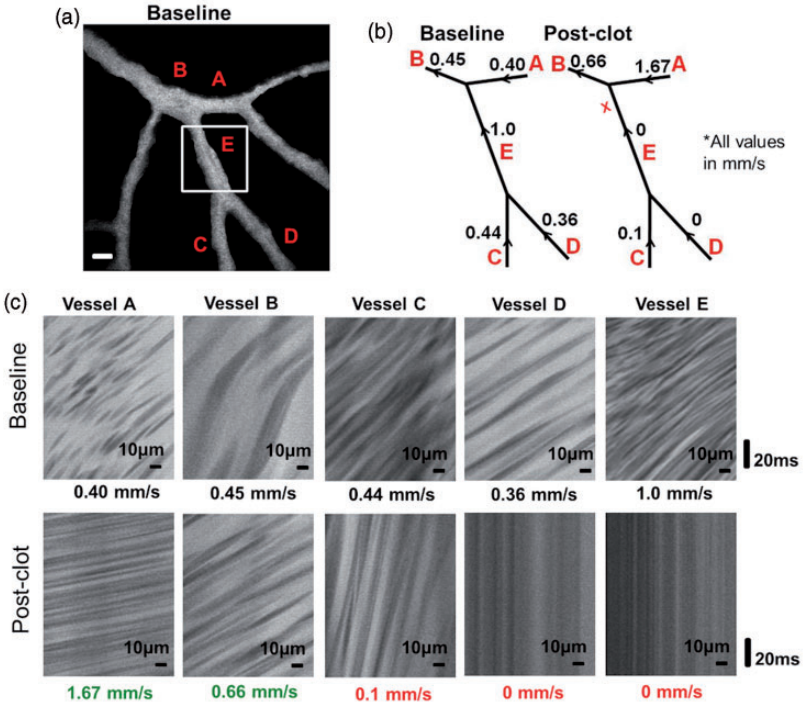

Figure 7 Blood flow velocities before and after the clot formation. a. Two-photon microscopy image showing the vitelline vein network of study. The white box indicates the clotted region. Scale bar is 200 μm. b. Measured blood velocity values in the vessels before and after the clot formation. c. Representative line scan pictures for each vessel. Increased flow is indicated with green and decreased flow is indicated with red

Table 1 Blood flow rates in the vessels of targeted network before and after the clot formation

Vessel	Flow rate before occlusion (10^{-3} mm^3/s)	Flow rate after occlusion (10^{-3} mm^3/s)
A	5.4	22.7
B	29.6	43.4
C	8.6	1.9
D	15.9	0
E	26.7	0

be seen clearly in the vessel before photodisruption (Figure 8a), after the insult, cellular debris fills the vessel and fills a clot (Figure 8b). Figure 8c is a brightfield image of the targeted network right after the insult while Figure 8d shows the network 24 h later. Comparison of the two images reveals marked changes in the structure of the network. Downstream vessels C and D are seen to degrade almost completely due to the complete flow cessation. The clotted vessel, vessel E partly degraded with no blood flow through it. Interestingly, upstream vessels A and B also partly degraded with no blood flow through them.

Discussion

Cardiovascular development is driven by both genetic and epigenetic factors and hemodynamic forces are thought to be important epigenetic stimuli. Deviations from normal hemodynamics were observed to affect cardiovascular development clinically, suggesting abnormal hemodynamic forces are important sources of CHDs. There are only few embryonic animal models of CHDs in which the blood flow is altered to reproduce the defective hemodynamics encountered in clinical observations. This is due to the scarcity of techniques that can be used to precisely control the severity of the induced defect. In the current study, a multiphoton microscopy-guided femtosecond-pulsed laser ablation system was utilized in order to study the vascular remodeling of embryonic blood vessels under altered blood flow environments. For this purpose, flowing blood inside vitelline vessels was irradiated to induce clot formation inside these vessels and the subsequent remodeling in the neighboring vessels under abnormal hemodynamics was investigated.

Most of the current *in-vivo* techniques to alter the hemodynamic environment for developing cardiovascular systems employ severe surgical procedures including bead insertion,[10] vessel banding,[15] chamber ligating,[12] and chamber clipping.[16] The significant results from these studies contributed substantially in the understanding of embryonic progression of CHDs. However, all of these techniques are invasive and induce global damage that are difficult to control and may lack clinically relevancy. Therefore, less invasive and better controlled techniques are needed.

Lasers provide a good alternative to these invasive techniques, because of the ability to precisely control the severity of the induced defect. Recently, Matrone *et al*. utilized a conventional laser system to induce micrometer-sized

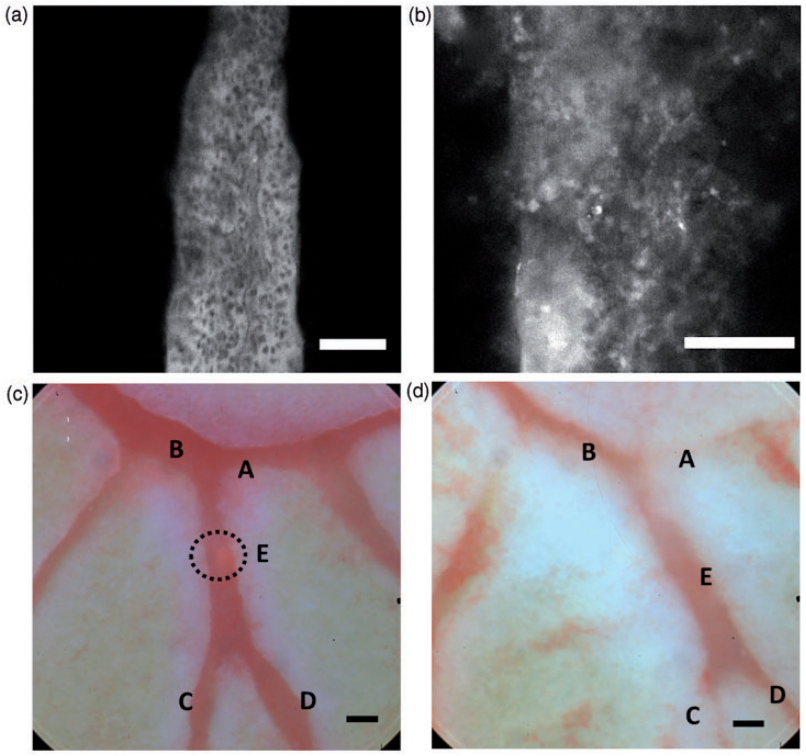

Figure 8 Cellular injuries for clot formation and vascular remodeling after the blood clot. a. Two-photon image of the targeted vessel before photodisruption. RBC can be seen clearly as black dots. b. Two-photon image of the photodisrupted vessel. Cellular debris forming the clot can be seen. c. A brightfield image of the network taken immediately after the clot formation at branch E. Bright clot region is marked with dotted lines d. A brightfield image taken 24 h after the clot formation, showing clear degradation of vascular structure due to altered blood flows. Scale bars in a and b 100 μm, in c and d are 100 μm

injuries onto the ventricles of zebrafish embryos in order to study myocardial repair mechanisms following injuries.[25] FPLP, is even superior to conventional lasers in preventing damage to the surrounding structures around the targeted zone. This method can deliver the laser energy, to a small volume (μm^3) through nonlinear optical absorption. This technique was previously used to induce well-controlled small size tissue defects onto AV cushions of chicken embryos to study the embryonic development of valvular heart defects.[21] These cushions function as prevalvular structures to ensure unidirectional blood flow. Ablation of a small tissue section on a cushion resulted in the disruption of the unidirectional flow which then led to the remodeling of AV valves and heart chambers similar to clinical cases. While hemodynamic abnormalities due to non-functioning valves can be seen in some CHDs, other major common characteristics are flow constrictions in blood vessels. The current work focuses on generating flow constrictions in embryonic blood vessels to disrupt hemodynamics. In the current study, a multiphoton microscopy-guided FPLP system was utilized in order to study the vascular remodeling of embryonic blood vessels under altered blood flow environments. Multiphoton imaging is a good option among *in-vivo* imaging techniques with high resolution and the ability to pass through the tissue of couple millimeters without photobleaching.[26] With the system that was utilized in the current study, as deep as 1.8 mm could be visualized, which was sufficient to image inside the vitelline vessels of HH24 embryos. Flowing blood inside vitelline vessels was irradiated with femtosecond laser pulses. At sufficient laser energies, the endothelial cells lining the vessel walls as well as the circulating RBCs in the blood were damaged. Cellular injuries resulted in a local micro-occlusion. It was possible to form a stable clot that constricts the blood flow completely when several micro-occlusions are formed inside the vessel (Figure 5). Clotting energy was found to increase linearly with the diameter of the vessel (Figure 6). To quantify blood flow rates in the neighboring vessels when a vitelline vessel is clotted, a vessel of about 200 μm diameter was clotted and the immediate vicinity was imaged. Blood velocities were measured via line scanning in the upstream and downstream vessels before and after the clotting procedure. Occlusion of blood flow resulted in an immediate dramatic increase of the blood velocity in the upstream vessels, and cessation of blood flow in the downstream vessels (Figure 7). Targeted and neighboring vessels were analyzed 24 h later. Downstream vessels were found to degrade almost completely, due to the complete flow cessation. The clotted vessel was also found to partly degrade with no blood flow through it. Interestingly, upstream vessels were also found to partly degrade with no blood flow through them. These results demonstrate that, vitelline vessels are very sensitive to

alterations in hemodynamic forces and remodel in response to load changes.

With the determined energy thresholds, this technique can be further used to clot a blood vessel inside the embryo or a vitelline vessel close to the heart for the purpose of studying the embryonic heart development under abnormal hemodynamic conditions. One limitation of the system is a maximum imaging and ablation depth of ~1.8 mm. Therefore, it is not possible to work with embryos older than HH26-27 with current settings.

In conclusion, multiphoton imaging-guided FPLP is a useful approach to visualize and alter embryonic cardiogenesis. While the current study concentrated on vitelline vessel hemodynamics, the methodology can be extended to any embryonic region. Techniques that will be developed to induce noninvasive perturbations to embryonic development will complement genetic and molecular screening tools. Utilization of these screening tools with novel microsurgery techniques will significantly contribute to reveal interplays between genetic and epigenetic factors that cause formation of CHD. Laser–tissue interaction knowledge gained from these studies will also contribute to the development of *in-utero* intervention techniques. One good example here is well-established fetoscopic laser therapy for placental twin-to-twin transfusion syndrome.[27]

Author contribution: HCY completed the design, execution, data analysis and manuscript preparation of the work.

ACKNOWLEGMENTS

The author would like to thank Prof Jonathan T Butcher and Akshay Shekhar from Cornell University, for driving him into researching embryonic development of congenital heart defects; Prof Chris B Schaffer and Prof Nozomi Nishimura from Cornell University for allowing him to use the laser setup; and Prof Vahan Kalenderoglu from Dogus University for reviewing the manuscript.

This research was supported by a Marie Curie International Reintegration Grant within the 7th European Community Framework Programme (IRG276987 to HCY) and by The Scientific and Technological Research Council of Turkey, TÜBİTAK (112M148 to HCY and 112M895 to HCY).

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(Received April 1, 2014, Accepted June 19, 2014)