4D shear stress maps of the developing heart using Doppler optical coherence tomography

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Abstract: Accurate imaging and measurement of hemodynamic forces is vital for investigating how physical forces acting on the embryonic heart are transduced and influence developmental pathways. Of particular importance is blood flow-induced shear stress, which influences gene expression by endothelial cells and potentially leads to congenital heart defects through abnormal heart looping, septation, and valvulogenesis. However no imaging tool has been available to measure shear stress on the endocardium volumetrically and dynamically. Using 4D structural and Doppler OCT imaging, we are able to accurately measure the blood flow in the heart tube in vivo and to map endocardial shear stress throughout the heart cycle under physiological conditions for the first time. These measurements of the shear stress patterns will enable precise titration of experimental perturbations and accurate correlation of shear with the expression of molecules critical to heart development.

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1. Introduction

Blood flow is a critical factor that regulates developmental programs during cardiogenesis. Alterations in blood flow during early cardiovascular development can lead to congenital heart defects (CHDs) [1–3]. Biomechanical forces exerted by the flow of blood likely influence gene expression in surrounding cells [4–7]. The altered gene expression then affects the form and function of the developing heart resulting in further alterations to the biomechanical forces. Due to the absence of appropriate tools to sensitively assess forces on the early looping heart this biomechanical feedback is poorly understood. Even small alterations in the heartbeat may influence blood flow that then results in altered levels and patterns of shear stress on the endocardium and potentially lead to abnormal heart looping, trabeculation, valvulogenesis and septation [3,8].

One critical biomechanical factor caused by blood flow and involved in heart development is the shear stress experienced by the endocardium. Shear stress in the developing heart is the force that is exerted on the endocardial cells by the blood dragging past them. Shear stress has been shown to be of critical importance in both controlling and regulating various cellular processes involved in heart development (reviewed in [4,9,10]). Alterations in the hemodynamic patterns in the developing heart have been shown to result in major cardiovascular defects including septal defects and outflow tract anomalies [1,8,11,12]. This is due in part to the wide variety of signaling molecules whose expression and activity are influenced by alterations in shear stress. Some of these proteins including KLF2, ET-1, NOS-3, and pERK which have all been shown to be influenced by shear stress and play a role in the developing avian cardiovascular system [3,6,9,13–15]. In order to better understand the connection between mechanotransducing molecules, blood flow-induced shear stress, and heart development, it is important for us to be able to accurately measure shear stress on the endocardium.

The ability to accurately measure biomechanical forces in the looping embryonic heart is complicated by the diminutive size of the heart at this stage (<2 mm) and the rapidly changing blood flow patterns. High-frequency pulse-echo ultrasound is commonly used to image fetal mouse hearts and to assess their cardiac function [16,17]. However, ultrasound imaging requires the transducer to be in acoustic contact with the sample and is not amenable to culture methods that maintain the early embryo in vitro under physiological conditions. Additionally, the resolution of ultrasound imaging is not sufficient to accurately measure...
developing hearts that are <2 mm in length. Previously, microparticle image velocimetry (µPIV) has been successfully used to estimate shear stress in the avian embryonic heart [18,19]. Unfortunately, µPIV is a 2D measurement and is unable to assess shear throughout the entire heart tube during cardiac looping. Optical coherence tomography (OCT) has shown great promise with regards to both structural and function imaging of embryonic heart development [20–27]. Van Leeuwen et al proposed a method to directly measure shear stress in vessels with resolution in depth using Doppler OCT [28]. Blood flow measurements and structural images acquired by OCT have also been used as boundary conditions for modeling shear stress patterns in the avian heart tube [29,30], and to measure the shear rate in chicken embryo vitelline vessels [25]. We have previously shown that by using Doppler OCT, shear stress can be measured at selected cross sections of the developing heart tube [31]. However, it is necessary to measure shear stress in 4D (i.e. 3D volumes in motion) in the developing heart tube, in order to thoroughly investigate the relationships between dynamic shear stress on the endocardium of the living, beating developing heart and molecular expression patterns regulating normal and defective developmental paths.

Here, we demonstrate a method based on 4D Doppler OCT to directly measure the blood flow-induced shear stress on the endocardium of early avian embryonic hearts over the course of a full heart cycle. 4D Doppler OCT image-sets of three individual embryonic hearts were acquired while they were incubated in an environmental chamber under physiological conditions [32,33]. Using image-based retrospective gating [26,34,35] we obtained 4D image data sets containing both structural and Doppler flow information. These data were used to create maps of shear stress on the endocardium at 14 time points during the cardiac cycle. This method for quantitatively mapping shear stress was verified using a capillary-tube flow phantom at multiple flow rates.

2. Material and methods

2.1. Embryo preparation

Fertilized quail eggs (Coturnix coturnix; Boyd’s Bird Company, Inc. Pullman, WA.) were incubated in a humidified, forced draft incubator at 38°C (G.Q.F. Manufacturing Co., Savannah, GA). After 48 hours of development the eggs were taken from the incubator, the eggshell was removed, and the contents were placed in a sterilized 35 mm Petri dish [36]. Once in the Petri dish, the surviving embryos were placed in an environmental OCT imaging chamber [37] with controlled temperature (38°C) and humidity to ensure imaging under physiological conditions.

2.2. OCT imaging

The OCT system used to collect the data utilized a buffered Fourier Domain Mode Locked laser as previously described [20]. The in-depth and transverse resolution was 8 μm and 10 μm, respectively, in tissue. 4D Doppler OCT data were collected by imaging over multiple heartbeats at sequential slice locations (Figs. 1B–1D) and reassembled using image-based retrospective gating [26]. 1000 A-scans were acquired per frame with a line rate of 117 KHz, and after reassembly a total of seventy volumes per heartbeat were acquired. A-scans were recorded at 1.4 μm steps in the B-scan direction. Data were also acquired from a calibration interferometer and used to resample the data evenly in wavenumber and to improve the Doppler signal by correcting for laser phase noise [31]. For each B-scan Doppler image a five-line rolling average was employed to reduce phase noise and phase wrapping was corrected using a Goldstein algorithm [38].

2.3. Shear stress measurements

In order to calculate the shear stress in the developing heart tube three assumptions were made. First, it was assumed that the blood is a Newtonian fluid with an approximate dynamic
viscosity, $\eta$, of 5 mPa s [18,31]. Second, it was assumed that the blood flow in the looping avian heart has both a low Reynolds number and a low Womersley number, indicating that the flow is laminar and dominated by viscous forces [18,31]. Finally, it was assumed that the blood moves in the direction of the center line of the heart tube.

The shear stress, $\tau$, was calculated using the equation $\tau = \eta \frac{du}{dn}$ [18] where $u$ is the fluid velocity parallel to the wall and $n$ is the radial distance from the surface of the tube. In order to find the velocity gradient normal to the wall $(du/dn)$ the endocardium was manually segmented at 14 evenly spaced time points throughout the cardiac cycle from the 4D OCT structural image data using image analysis software (Amira, Visage Imaging). The segmented endocardial surfaces were employed to determine the centerlines of the heart tube at each time point (Fig. 2A). The centerlines were originally calculated within Amira using a TEASAR (tree-structure extraction algorithm for accurate and robust skeletons) [39] algorithm and then smoothed using custom analysis software (MATLAB, MathWorks). Assuming the blood is all moving in the direction of the center of the heart tube, tangent lines at each point along the centerline were calculated and then used to correct the Doppler OCT data to estimate the absolute blood velocity at each time point. The segmented endocardium was then used to generate an outer surface shell representing the endocardial wall on which the shear stress will be calculated. The surface shell was a mesh composed of 4,000 connected triangular faces and the direction normal to each triangle was calculated (Fig. 2B). This direction was used to determine the velocity gradient from the endocardial wall, which was computed as the local slope of the velocity profile (within 45 $\mu$m of the endocardial wall). The shear stress values were then calculated by the formula above and plotted using a color scale on the corresponding surface mesh. Paired Student’s $t$-tests (Excel, Microsoft) were performed on peak shear stress values in various regions, and statistical significance was achieved when $p<0.01$.

2.4. Phantom validation experiment

In order to verify the method of shear stress measurement, a capillary tube phantom was created to simulate the flow through the heart with a controlled velocity profile. The capillary tube had an inner diameter of 0.5 mm and was perfused with a 2% lipid suspension solution (Intralipid) solution using a syringe pump. 4D Doppler OCT data were obtained at five different flow rates ranging from 0.25 ml/min to 2 ml/min. The syringe pump flow rates were calibrated by measuring the total volume of Intralipid solution pumped during a specific period of time using a graduated cylinder. At each flow rate, the Doppler data were then used to calculate wall shear stress at 7 cross-sectional slices utilizing the methods described above. The average shear rate on the inner wall of the capillary tube was then compared with the shear rate calculated using the known flow and tube geometry.

3. Results

For this demonstration, stage HH13 quail embryos ($n=3$) were cultured using a shell-less culture method and imaged using OCT as described in detail in the Methods section. Stage HH13 embryos were selected because cardiac looping is occurring during this stage and these embryos exhibit dramatic morphological changes during this developmental time period. OCT not only allows clear observation of these morphological changes, but it is also capable of visualizing the internal anatomical structures of the developing avian heart in detail as seen in Fig. 1.

4D Doppler OCT data sets were assembled as described in detail previously [31] and summarized in the Methods section. These image sets include both structural and Doppler flow velocity data, as shown in Fig. 1. Extracting endocardial wall shear stress (WSS) from these data requires significant analysis. Under assumptions detailed above, the WSS is proportional to the blood velocity gradient in the direction normal to the wall of the endocardium, known as shear rate, and to the blood viscosity. Determining the shear rate at
Fig. 1. Panel A shows a quail embryo imaged with a stereomicroscope at 12X magnification. This embryo was removed from the yolk and inverted using the New culture for clear visualization under the microscope. All other embryos imaged by OCT in this work were left on the yolk as described in detail in the Methods section. Panel B shows a cross sectional image of the quail embryo heart imaged by OCT. The cross section was recorded at approximately the location of the green dotted line in panel A. Imaging by OCT allows for the visualization of the myocardium, cardiac jelly, and endocardium in vivo in both the inflow and outflow region of the heart tube. Panels C and D show Doppler OCT data overlaid on a structural cross section of the outflow tract of the heart tube during diastole and systole respectively at the approximate location of the red dotted line in panel B. The increasing red color represents increasing blood velocity in the forward direction and the blue represents retrograde blood flow as represented by the color bar. Myo, myocardium; CJ, cardiac jelly; BL, blood; Endo, endocardium.

Fig. 2. Panel A shows the centerline (pink curve) through the segmented endocardium of a representative heart tube during diastole. Tangents to the centerline were used to determine the Doppler angle for absolute velocity calculations. Panel B shows the surface mesh of a representative segmented endocardium of a heart tube cut at the location of the dotted line in panel A. The white arrows pointed inward along the surface mesh represent the normal vectors to the endocardium along the entire inner wall of the heart tube.

Each point within the heart tube required: (a) the location of the entire surface of the endocardium, which was obtained by segmenting the structural OCT images, and (b) the blood velocity profile, which was obtained from Doppler OCT, corrected by assuming that the blood flows in the direction of the center line of the heart tube (Fig. 2). Blood viscosity was assumed from previously published work [19].

Shear stress maps were calculated at 14 evenly-spaced time points during the cardiac cycle of each embryo. Examples of four time points from one embryo are displayed in Fig. 3 (more data are shown in Media 1). The shear maps show shear stress values as high as 7.7 Pa in the
outflow segment of the heart tube compared with a maximum at the inflow segment of 3.1 Pa during the course of the heartbeat. Higher shear stress is also apparent on the inner curvature of the heart tube compared to the outer curvature (Fig. 4). These observations were evident in all three heart tubes mapped. A segment in the middle of the heart tube (marked in gray in Fig. 3) was not analyzed because the blood flow in this area is nearly perpendicular to the OCT imaging beam, leading to little or no Doppler signal. As a result, accurate blood velocity measurements were not obtained in this area. Currently, we are primarily interested in the inflow and outflow segments of the heart because these are the location of future cushion and valve development, and because data from previous studies are available in these regions for verification [30]. However, by recording 4D Doppler OCT image sets with the scanner oriented at different incidence angles, a complete flow map of the entire heart tube can be generated.

Fig. 3. Shear stress on the endocardium. Shear stress is calculated using the velocity gradient normal to the wall of the heart tube and the viscosity of blood. Four evenly spaced time points during a heart cycle are represented and the shear stress values are displayed on the endocardium surface. The represented heartbeat lasted 367 ms. The gray region represents the area where valid Doppler OCT data were not obtained because the direction of the blood flow was nearly perpendicular to the OCT imaging beam. See also Media 1.

4D shear stress maps enable visualization of the shear stress patterns at specific areas of interest in the developing heart (Fig. 4). 3D maps representing each time point during the cardiac cycle may be examined from multiple orientations. In particular, the outflow tract of the heart tube was examined at the time of highest shear stress from various viewing angles (Figs. 4B and 4C). Higher shear stress was consistently observed on the inner curvature of the outflow tract when compared with the outer curvature in all three hearts examined. This trend was also consistent over the course of the entire heart cycle.

In addition to 3D spatial shear stress maps, this technology also provides temporal shear stress information as shown in Fig. 5. Here, shear stress traces at three locations on the endocardial wall are shown over the full heart cycle. The shear stress traces clearly show significant differences in both the magnitude and the shape of the waveform depending on the location in the heart, with the inner curvature of the outflow tract displaying the highest peak shear stress values. At point A in Fig. 5 (inner curvature) the maximum shear stress is approximately four times the peak shear stress at point B (outer curvature). Negative values of
Fig. 4. Shear stress on the inner and outer curvature of the outflow tract. Panel A shows the 3D shear stress map at the time of maximum shear stress in the outflow tract. Panel B and C show the shear stress map of the same heart cropped to show only the outflow tract. Panel B shows the shear stress map oriented to view the outer curvature of the heart tube and Panel C shows the shear stress map oriented to view the inner curvature of the heart. The viewing direction is represented in panel A by the two arrows.

Fig. 5. Shear stress measured over time. Panels A-C shows the measured shear stress over time at three different locations in the same heart, namely the inner and outer curvatures of the outflow tract, and the inflow tract, respectively. The shear stress was calculated for the duration of one effective heart cycle and displayed three times for ready visualization. The locations represented by all three traces are indicated in the 3D surface mesh shown in panel D. P, pumping phase; F, filling phase.

Shear stress (e.g., Fig. 5A) indicate regurgitant flow, which is common in the outflow tract at this stage of development. The inflow trace (Fig. 5C) shows a double peak pattern that is also observed in pulsed Doppler traces of the inflow tract and in the venous system in general. This trace shows less shear stress associated with the pumping phase of the heart cycle (the first peak), and higher shear stress associated with the filling phase (the second peak).
The maximum shear stress values in 3 different embryonic quail hearts at HH13 (Table 1) at the inflow tract and the inner and outer curvature of the outflow tract are shown in Table 1. The patterns of peak shear stress in all three embryos were found to be very similar. The average peak shear stress was found to be 7.7 Pa on the inner curvature of the outflow tract, 2.0 Pa on the outer curvature of the outflow tract, and 3.1 Pa on the inflow tract. The inner curvature of the outflow exhibits significantly higher peak shear stress than the outer curvature ($p = 0.003$) or the inflow ($p = 0.005$).

Table 1. Maximal shear stress at the inflow and outflow regions of the heart (Pa)$^a$

|          | Max Inflow | Max Outflow | Max Outflow |
|----------|------------|-------------|-------------|
| Heart 1  | 3.0        | 7.4         | 2.0         |
| Heart 2  | 3.1        | 8.1         | 1.6         |
| Heart 3  | 3.4        | 7.6         | 2.5         |
| Average  | 3.1 ± 0.1  | 7.7 ± 0.1   | 2.0 ± 0.2   |

$^a$IC, inner curvature; OC, outer curvature.

In order to verify the accuracy of this new method of shear stress measurement, a phantom experiment was performed under known flow conditions. A syringe pump forced a 2% Intralipid solution through a straight capillary tube with an inner diameter of 0.5 mm. A segment of the tube was imaged by 3D Doppler OCT at 5 different flow rates controlled by the syringe pump. At each of the 5 different flow rates, shear rate on the inner wall of the capillary tube was measured at seven locations on the tube. Because the flow was constant and the tube was straight, the shear rate on the tube wall was expected to be uniform, so that variability between the seven measurements would represent the measurement precision. The range of flow rates were selected to cover the range of shear rates experienced within the heart tube at this stage of development. The results are summarized in Fig. 6. The dotted line represents the peak shear stress value measured using this method in the embryonic heart tube. The solid line indicates the theoretical shear rate based on the flow values assuming a laminar flow profile. The measured values deviated from the theoretical value by an average of 2% across all flow rates. The precision of multiple measurements at each flow rate was 2% as estimated by relative standard deviation (the ratio of standard deviation to the mean). These

![Fig. 6. Shear rate measurement verification. The x-axis represents the actual flow rate recorded from the syringe pump. The y-axis shows the shear rate values measured from the Doppler OCT data taken at each flow rate. These calculations were repeated for 7 experiments at each flow rate. The solid line represents the theoretical shear rate based on the measured flow rates. The dotted line represents the peak shear stress value measured in the embryonic heart.](image-url)
results are reported in terms of shear rate rather than shear stress because shear rate is the fundamental measurement obtained from the Doppler OCT data. Viscosity is assumed to be a constant scaling factor, and the viscosity of Intralipid solution (~1 mPa s) differs significantly from that of embryonic blood at this stage of development (~5 mPa s).

4. Discussion

OCT derived 4D maps allow direct measurement of the shear stress at any region of interest in the developing heart, which is a significant advance over previous 2D measurement techniques. The shear stress values presented here correspond well with those reported previously in specific regions of interest. The peak outer curvature shear stress values obtained by 4D OCT (1.6-2.5 Pa) are similar to those reported by μPIV on the top surface of the outflow tract of a HH 17 stage chicken heart (1-3 Pa) [19]. Also, OCT shear stress values at the inner curvature (7.4-8.1 Pa) are similar to those estimated with finite element modeling of the outflow tract of a HH18 chick embryo (11 Pa) [30]. These associations are encouraging, but exact correspondence is not expected because of differences in animal models, developmental stages, and model preparation methods.

The shear stress measurements presented here made use of three assumptions that have the potential to introduce uncertainty in the shear stress values. One assumption is that of the blood viscosity at this stage of embryonic development. The value of 5 mPa s has been used in previous works [18,31] and we believe it to be a reasonable estimate. However, in this shear stress measurement, the viscosity merely serves as a scaling factor. Therefore, the comparisons between different regions of the heart are still valid regardless of the value of the blood viscosity. Another assumption is that the blood flow at this stage of embryonic heart development is dominated by viscous forces, which leads to laminar flow. Previous groups have shown the Reynolds number to be approximately 0.5 in similarly staged embryos which is well below the threshold needed for the development of turbulent flow (<1000-2000) [18]. Additionally, the Womersley number at this stage of development is on the order of 0.2 which allows for the safe assumption of a parabolic velocity profile (<1-2), which is also observed in our OCT data [17,18]. Finally, we assume that the blood is flowing in the direction of the heart tube’s centerline. This is a reasonable assumption for the same reasons stated above. Were this not to be the case, it would influence our estimations for the absolute velocity of the blood at each cross sectional location. An error in the Doppler angle estimation would impart a proportional error in the absolute velocity measurements. Because such an error would be small, and would not change quickly, there is a potential to cause uncertainty in comparing measurements taken from regions of the heart that are far from each other (e.g. inflow tract and outflow tract), but it is unlikely to influence the comparison of measurements taken in close proximity to each other (e.g. inner curvature and outer curvature).

This work represents the first report of shear stress measured by the use of imaging in 3D throughout the cardiac cycle. 4D shear maps allowed comparison of shear values at one region of the endocardium to another region within the same heart. Higher shear stress was observed on the inner curvature in the outflow tract of the developing heart compared with the outer curvature. This trend corresponds well with previously reported results obtained through the use of modeling [10,30] and μPIV measurements [19]. The higher shear stress values on the inner curvature in the outflow tract is interesting due to the fact that the outflow tract is where the future aortic and pulmonary valves and septae will develop [40]. It has also been shown that changes in flow and therefore shear stress may facilitate atroventricular valve development [3]. 4D shear mapping will be useful for future investigations of the relationship between hemodynamics and valve development in specific areas of the developing heart tube.

4D OCT shear mapping is expected to significantly benefit the investigation of early heart development, but some limitations remain that can be overcome in the future. One limitation is that there are manual processing steps necessary to analyze the image data sets including endocardium segmentation and phase unwrapping. This makes it time-consuming to process
the results of experiments that involve a large number of embryos. Automated image processing algorithms are in development in order to enable high-throughput experiments using 4D data sets from larger numbers of embryos, particularly automated segmentation of the endocardium boundary. A limitation of the presented imaging protocol is that Doppler OCT signal is not obtained from the center of the heart tube. This is due to the fact that the blood flow in this segment is nearly perpendicular to the OCT imaging beam. This limitation can be overcome simply by recording an additional 4D image set with a different angle of incidence. Multi-beam Doppler OCT methods have also been demonstrated that can image from multiple orientations in one shot [41,42].

The combination of Doppler OCT-derived shear stress and molecular staining will enable new investigations to better understand the role of shear stress in the development of the heart tube. Doppler OCT allows analysis of shear stress at each moment during the cardiac cycle. These measurements will allow for the precise localization of highest shear stress both spatially and temporally without relying on modeling. Using Doppler OCT the change of shear stress over time can also be analyzed to determine metrics such as the oscillatory shear index (OSI) at specific locations within the heart tube. Abnormal OSI has been shown to have a close correlation with abnormal valve formation in zebrafish [3]. This metric could prove interesting and valuable for investigations of the effects of changes of regurgitant flow on the development of the heart. Additionally, future experiments could involve perturbing flow in the developing heart via drug intervention or optical pacing [43]. The resulting altered hemodynamics would be difficult to model, whereas direct measurement of the shear stress with Doppler OCT is straightforward. These measurements could then be correlated with expression levels shear stress responsive markers to gain a better understanding of exactly which cells are most affected by the altered shear stress. Together these complementary tools may prove to be powerful for future investigations into the role of shear stress in signaling the development of the heart.

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