Valveless pumping mechanics of the embryonic heart during cardiac looping: pressure and flow through micro-PIV

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Submitted as an Original Article

Keywords: heart, zebrafish, blood flow, pressure, development

Number of words: 3081

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ABSTRACT

Cardiovascular development is influenced by the flow-induced stress environment originating from cardiac biomechanics. To characterize the stress environment, it is necessary to quantify flow and pressure. Here, we quantify the flow field in a developing zebrafish heart during the looping stage through micro-particle imaging velocimetry and by analyzing spatiotemporal plots. We further build upon previous methods to noninvasively quantify the pressure field at a low Reynolds number using flow field data for the first time, while also comparing the impact of viscosity models. Through this method, we show that the atrium builds up pressure to ~ 0.25 mmHg relative to the ventricle during atrial systole and that atrial expansion creates a pressure difference of ~ 0.15 mmHg across the atrium, resulting in efficient cardiac pumping. With these techniques, it is possible to noninvasively fully characterize hemodynamics during heart development.

1. Introduction

As the first developing organ, the heart produces flow- and pressure-derived mechanical stress that provides critical feedback to cardiovascular cells in the form of mechanotransduction (Bark Jr et al., 2013; Helmlinger et al., 1991; Hove et al., 2003; Lucitti et al., 2007; Vermot et al., 2009; Yashiro et al., 2007). Zebrafish have been useful model for studying genetics and functional aspects of heart development in relation to flow since they are transparent and develop similarly to other vertebrates including humans through looping stages (Hove et al., 2003; Johnson et al., 2013b; Stainier et al., 1993; Vermot et al., 2009). In this model, the embryo forms a valveless primitive linear heart tube structure by ~24 hours postfertilization (hpf) (Forouhar et al., 2006; Liebling et al., 2006; Stainier et al., 1993). Chambers in the heart begin to form during cardiac looping (tube bends) at approximately 36 - 48 hpf, a stage that is sensitive to prior alterations in mechanical loading (Bakkers, 2011; Hove et al., 2003; Johnson et al., 2015). Eventually the heart develops a valve at the atrioventricular canal (AVC) after ~100 hpf (Liebling et al., 2006; Vermot et al., 2009). However, studies relating the influence of biomechanics on these developmental stages remain limited due to difficulties in quantifying the pressure field. Pressure produces stress in the heart wall, which provides a critical signal for cardiovascular modeling and remodeling (Bark Jr et al., 2013; Fung, 1981; Helmlinger et al., 1991; Taber, 2001). Cannulae have been used for direct pressure measurements at single points (Hu et al., 2000; Kopp et al., 2005), but this approach remains highly invasive and due to the single point of measurement, it provides little insight into the distribution of pressure in a single heart. Laplace's Law has been used to noninvasively estimate the pressure gradient in the primitive linear heart tube (Forouhar et al., 2006). However, this method requires many assumptions and knowledge of circumferential stress. Our group has also used a highly simplified analytical model to demonstrate the role of chamber contractile patterns in driving reverse flow through the pressure gradient (Bulk et al.). This work required many simplifying assumptions, such as neglecting viscous shear stress, and only provided limited qualitative details of the pressure difference between two points. There remains a need to noninvasively fully quantify the pressure field of the heart to quantify relative stress in the wall and to better link contractile mechanics to pressure that drives flow.

Here, the objective of our work is develop and apply a noninvasive technique to quantify the relative spatial distribution of pressure, or the pressure field, in the developing heart using only the flow field calculated through micro-particle imaging velocimetry (μ PIV). Although two primary types of post-processing methods have been developed along these lines to study pressure, neither has been employed to study the low Reynolds number (Re) flow during cardiovascular development to the knowledge of the authors. In the first technique, a form of the Poisson equation is solved. This method is prone to implementation challenges when defining boundary conditions and is sensitive to the choice of a Laplacian operator (Dabiri et al., 2013; Golub and Van Loan, 2012). As a result, others have directly integrated the pressure gradient in the momentum equation (Baur and Köngeter, 1999; Dabiri et al., 2013; Liu and Katz, 2006):

$$\partial_i P = -\rho \left(\frac{\partial u_i}{\partial t} + \partial_j u_j u_i \right) + \mu (\partial_k \partial_k u_i), \quad (1)$$

where P is pressure, ρ is the fluid density, u_i is the i^{th} component of the velocity vector, and μ is the dynamic viscosity. This method has been proven accurate for moving boundaries in biological flow, but has been primarily used for high Re flows (Dabiri et al., 2013; Liu and Katz, 2006, 2013). Therefore, we build upon these techniques for low Re internal flow in the present work. Further explanation of techniques that leverage flow field data to quantify pressure are discussed in the review by van Oudheusden (Van Oudheusden, 2013). Here, we use Eq. 1 to quantify the relative pressure field in the developing zebrafish heart during cardiac looping. We further validate the technique and consider multiple viscosity models that could be relevant to blood flow during development.

2. Methods

2.1. Zebrafish Handling and Imaging

Embryos are obtained from fertilized eggs collected from adult wild type zebrafish (*Danio rerio*) that are raised and bred in accordance with Westerfield (1995) with experiments approved through IACUC at

Colorado State University (Westerfield, 1995). Embryos are incubated at 28°C, dechorionated at approximately 24 hpf, and imaged at 48 hpf under a brightfield stereo microscope (Olympus SZX12 with SZX-AL20X lens attachment). Embryos are imaged ventral-side up in a low gelling temperature agarose (Sigma Aldrich, Type VII-A, A0701), as previously described with an attempt to image along a cross-section aligned with the orientation of the heart (Johnson et al., 2015). The setup is illustrated in the supplementary material as Fig. SI-1. All imaging is performed at the midplane of the atrium, determined after focusing on each cardiac wall perpendicular to the imaging plane. Images are acquired at 1500 frames per second at 0.95 $\mu\text{m}/\text{pixel}$ using a high speed camera (Photron FASTCAM SA3) with a resolution of 0.88-1.5 μm . A background image is subtracted from all images of the zebrafish heart after registration and intensity normalization, with a sample preprocessed image shown in Fig. 1A with labeled anatomical features, and a post-processed image in Fig. 1B. The background image is calculated as an ensemble average based on the median of 5 cardiac cycles, resulting in a separate background image for each point in the cardiac cycle, a technique different from the sliding average previously used (Jamison et al., 2013; Johnson et al., 2013a). Therefore, the heart wall is removed, but slow moving red blood cells (RBCs) are not filtered from the images when performing flow calculations. We are presenting representative data from 19 experiments.

2.2. Flow Analysis

2D μPIV is used to quantify the entire flow field of the atrium through DaVis 7.2 software (Lavision, Germany). μPIV is performed for every $1/10^{\text{th}}$ portion of the cardiac cycle period (T). For each of the 5 cycles, the mean velocity field is derived from the cross correlation of 2 image pairs. An ensemble average of the velocity field is taken at fraction of T for 5 cardiac cycles. A mask is used to isolate flow from any noise created from background subtraction, Fig. 1C. Cross-correlation vectors are obtained using an initial pass of a 32x32, followed by a 16x16, 12x12, with a final pass of an 8x8 pixel interrogation window, with 50% overlap. The final pass interrogation window approaches the size of

RBCs, which are used as the seeding particle, similar to other groups (Hove et al., 2003; Jamison et al., 2013; Lee et al., 2007). This interrogation window size is chosen to balance our signal-to-noise ratio, while trying to resolve cellular scale flow patterns in the heart. Based on previous studies, the use of RBCs in μ PIV can result in an underestimation of the centerline velocity by an average of 25% for a chick embryo with an error that increases with increasing velocity (Poelma et al., 2012). However, to minimize the invasiveness of our technique, we choose to image erythrocytes in the absence of a separate tracer particle. Furthermore, our phase-based subtraction technique increases the accuracy of μ PIV by removing background bias, as previously shown (Jamison et al., 2013).

2.3. Pressure Analysis

The pressure field for this study is quantified by integrating Eq. 1 for a known velocity field that is interpolated for pressure integration paths. Central differencing schemes were used in velocity gradient calculations. Pressure is then integrated along the entire fluid-solid boundary for an initial pressure estimate while setting a single boundary node to a pressure of 0. Since a single integration path can result in the accumulation of error, we subsequently integrate along multiple paths. Each path begins at a node and ends at a fluid-solid boundary. Paths extend in an array of circumferentially uniform directions specified by the angle between each path, as illustrated in Fig. 1D. Integration paths are subsequently averaged, similar to previous studies (Dabiri et al., 2013; Liu and Katz, 2006, 2013). The technique is possible since each integration path is an independent estimate of the pressure and uncorrelated measurement errors since pressure is a scalar property, as discussed in Dabiri et al. (Dabiri et al., 2013). Similar to Liu and Katz, we use the mean value of multiple integration paths (Liu and Katz, 2006, 2013). The mean value is updated in multiple iterations until the pressure field converges to a solution, as defined by a maximum pressure residual of less than 1×10^{-3} . To conserve computational effort, we first solve the pressure field at the fluid-solid boundary and then solve the pressure field in the fluid domain

after already obtaining accurate pressure boundary conditions. A first order upwind spatial integration method is used for the pressure integration.

2.4. Blood Viscosity

The viscosity model used in Eq. 1 can impact the pressure, but viscosity of blood in the embryonic zebrafish remains ill-defined. Values have been reported at 8 cP (~2x human blood) for trout and applied in zebrafish (Lee et al., 2013; Sørensen and Weber, 1995). However, blood is known to change with the local shear rate, the channel size, and the stage of embryonic development (Cho and Kensey, 1991; Fung, 1981; Pries et al., 1994; Santhanakrishnan and Miller, 2011). Similar to others we apply human models of viscosity to determine the potential impact on relative pressure calculations. We further apply an avian Newtonian model for a hematocrit of 15-17% since the blood is nucleated, similar to the blood of zebrafish (Al-Roubaie et al., 2011; Santhanakrishnan and Miller, 2011).

3. RESULTS

3.1. Fluid Velocity

Velocity profiles across the AVC and atrial inlet are shown in Fig. 2 with an additional discussion in the supplementary material. The maximum speed of flow through the AVC is 5 mm/s, corresponding to a $Re = \langle v \rangle D / \nu$ of 0.017 and a Womersley number, $\alpha = D(\omega/\nu)^{1/2}$ of 0.045, assuming a viscosity of 8 cP, a density of 1.025 kg/L, and $\omega = 2\pi HR$. Atrial systole continues for approximately $1/3^{rd}$ of the cycle as the atrium empties and the ventricle is filled. At the start of atrial diastole (0.3T), there is very little initial flow at the atrial inlet and no flow across the AVC, which is now closed. At 0.5T, velocity through the atrial inlet peaks at ~3 mm/s, corresponding to an inlet diameter of ~30 μm , which results in a Re of 0.012 and a α of 0.015. Overall, atrial diastole continues over $2/3^{rd}$ of the cardiac cycle as the atrium fills with blood.

3.2. Pressure

The velocity data is used to compute the pressure field presented in Fig. 3-5. Validation of the pressure algorithm used for these figures is provided in the supplementary material relative to grid resolution, number of integrations paths, and noise. Since the Re and α are low, the first 2 terms on the right hand side of Eq. 1 are negligible compared to viscous term. Therefore, pressure in Fig. 3-5 is normalized by the HR , which is the characteristic time scale, and viscosity, which is ill-defined in the developing zebrafish heart.

The atrial inlet is nearly closed at the start of atrial systole ($0.1T$), Fig. 3. A 0 pressure boundary condition is supplied at the sinus venosus (SV), corresponding to the venous return. Blood pressure in the atrium is approximately the same as the SV, while blood pressure in the ventricle becomes negative, $-500 HR*\mu$. Closure of the atrial inlet produces a local high pressure, $\sim 250 HR*\mu$, which pushes blood bidirectionally away from the inlet, but the majority of blood is driven in the direction of the ventricle with combined ventricular expansion and atrial contraction.

As atrial systole continues, the atrial inlet is closed and the AVC is open, as seen in Fig. 4. A 0 pressure value is set at the ventricle. Therefore, it should be noted that all pressure values are relative to the lowest pressure in the ventricle and that pressure during atrial systole in Fig. 4 should be viewed independently of other figures. During atrial systole, the pressure in the atrium is higher than the pressure in the ventricle ($0.15T$). This pressure difference increases to greater than $1500 HR*\mu$ ($0.20T$) and then decreases, until the atrium is nearly emptied ($0.25T$). Overall, the pressure in the atrium remains relatively uniform, with a large pressure drop across the AVC and a slight pressure gradient seen in the ventricle ($<750 HR*\mu$).

Atrial diastole begins around $0.3T$, with the expansion of the atrial lumen beginning at the atrial inlet, which continues as a wave to the AVC, Fig. 5. Pressure in the atrium is negative relative to the SV with a gradient in pressure from the atrial inlet to the leading edge of the expanding wave ($0.4-0.8T$), Fig. 5. Pressure in Fig. 5 is all relative to 0 at the SV, which is assumed to remain constant throughout atrial

diastole. The largest pressure gradient occurs around 0.5T, also corresponding to maximum speed of blood across the atrial inlet, as shown in Fig. 2. Subsequently, the pressure gradient begins to decrease and blood across the inlet slows down. Pressure in the atrium becomes relatively uniform, while remaining lower than the SV (0.7-0.8T), causing blood to continue entering the atrium at the inlet, while flow becomes stagnant near the AVC. Eventually, the pressure in the atrium equilibrates with the pressure in the SV, as the atrium becomes filled with very little blood flow (0.9-0.0T).

3.3. Viscosity Effects on Pressure

Pressure values depend on the chosen viscosity model, which can depend on the shear rates that are given in Fig. 6A for the entire cardiac cycle in the form of a box plot. The corresponding pressure drop across the atrial inlet and the AVC are shown in Fig. 6B for each viscosity model given in Table 1 and Fig. 6C. During atrial systole, the mean shear rate is on the order of $10\text{-}100\text{ s}^{-1}$, which corresponds to a highest viscosity predicted in the Newtonian 8 cP model with a predicted pressure drop of 0.25 mmHg and the lowest predicted in the 1.2 cP model with a pressure of ~ 0.04 mmHg across the AVC. At lower shear rates during atrial diastole, the pressure drop ranges from 0.02-0.16 mmHg with a maximum drop and viscosity seen for the power law model. Overall, the pressure drop varies with the chosen viscosity model, but remains within an order of magnitude.

4. DISCUSSION

Relative pressure is noninvasively quantified during the looping stage of a developing valveless zebrafish heart. By calculating pressure, we show that atrial contraction and ventricular expansion work in concert to minimize retrograde flow at the beginning of atrial systole, therefore increasing the efficiency of the heart. Specifically, ventricular expansion creates a negative pressure value supporting suction as the atrial inlet contracts. Subsequently, the atrium creates a relatively high uniform pressure head that pushes blood into the ventricle, working as a compressing closed vessel, similar to a balloon. It is possible that

the simultaneous expansion of the ventricle is helping to drive flow, but to the knowledge of the authors, it remains impossible to quantify the relative influence on pressure without a known pressure boundary condition that could otherwise demonstrate negative pressure in the ventricle. The pressure field for flow from the atrium to the ventricle is distinctly different from the pressure field for flow across the atrial inlet during atrial diastole, which appears to be driven by a pressure gradient caused by an expanding wave of the endocardium. Myocardial relaxation may support expansion, which likely requires coordination between the myocardium, cardiac jelly, and endocardium (Barry, 1948). Overall, with the ability to quantify the pressure field, it is possible to gain insight into pumping mechanics. This technique can be combined with physical pressure measurements to quantify absolute pressure as opposed to relative pressure. It can also be simply extended to a 3D flow field by including the 3rd coordinate in the momentum equation.

Quantitatively, we show that the pressure drop across the AVC can reach ~0.04-0.25 mmHg during atrial systole. In comparison, Lee et al. found a drop of ~0.4 mmHg at the AVC through CFD during a similar developmental stage using a viscosity of 8 cP (Lee et al., 2013). This value is comparable to absolute pressure values measured at a single point in the zebrafish ventricle (Hu et al., 2000; Kopp et al., 2005). Chick embryos exhibit a peak pressure drop of ~0.2-0.3 mmHg across the AVC, based on extrapolation (Hu and Clark, 1989; Hu and Keller, 1995). In comparison with the literature, our pressure estimation matches closely with physically measured values, with lowest values predicted when assuming a near-plasma viscosity of 1.2 cP.

Blood velocity in the current study reaches 6-7 mm/s at the AVC and ~3 mm/s at the atrial inlet, resulting in shear rates on the order of 10-100 s⁻¹. Blood velocity is similar to the literature and shear rate values on the same order as wall shear rates reported at the ventricle outlet (Hove et al., 2003; Jamison et al., 2013; Johnson et al., 2013a). However, it should be noted that our shear rate calculations are limited in spatial resolution and therefore actual wall shear rate values may be higher than those we report in Fig. 6B. Velocity profiles in Fig. 3B were blunted during atrial diastole and were slightly blunted during atrial systole. The blunted profile may be in-part due to the influence of a relatively large interrogation window

size (7.8x7.8 μm). Therefore, potential velocity vectors in that region are averaged. The profile may also be influenced by the hematocrit and low shear rate, which are known to support blunted profiles in the adult human (Fung, 1981). The blunted profile is unlikely due to temporal or spatial variations in geometry since the Reynolds number and Womersley number is low.

The current study is limited to planar images of the heart, which is further discussed in the supplementary material. Flow is calculated from the motion of cells as opposed to a direct measure of the surrounding plasma. Therefore, the cell may lag or lead the bulk fluid flow in the heart, providing possible errors in calculating the pressure field. Imaging for the current work is limited to the atrium because the eyes and head of the zebrafish block the view of part of the ventricle and distal vasculature. These components could be visualized by changing sample orientation, but then it is no longer possible to image the mid-plane of the atrium.

Acknowledgements

We acknowledge funding from the National Science Foundation (Award# 1235305), program director Dr. David Fyhrie

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336 **Tables**

Blood Model	Equation	Constants
Newtonian 8cP*	$\mu = \mu_{\infty}$	$\mu_{\infty} = 8 \text{ cP}$
Casson†	$\mu = \frac{(\sqrt{\tau_y} + \sqrt{\eta\dot{\gamma}})^2}{\dot{\gamma}}$	$\eta = 4 \text{ cP}, \tau_y = 5 \text{ cP/s}$
Carreau†	$\mu = \mu_{\infty} + (\mu_0 - \mu_{\infty}) \left[1 + (\lambda\dot{\gamma})^2 \right]^{\frac{n-1}{2}}$	$\mu_{\infty} = 3.45 \text{ cP}, \mu_0 = 56 \text{ cP},$ $\lambda = 3.313 \text{ s}, n = 0.3568$
Power Law†	$\mu = \begin{cases} m(\dot{\gamma})^{n-1} & \dot{\gamma} < 427 \\ \mu_{\infty} & \dot{\gamma} \geq 427 \end{cases}$	$\mu_{\infty} = 3.45 \text{ cP}, m = 35 \text{ cP*s}^{-0.6},$ $n = 0.6$
Newtonian 1.2 cP‡	$\mu = \mu_{\infty}$	$\mu_{\infty} = 1.2 \text{ cP}$

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338 Table 1: Viscosity models taken from the literature *(Sørensen and Weber, 1995), †(Cho and Kensey,
339 1991), and ‡(Al-Roubaie et al., 2011)

Figure Legends

Figure 1: A) A raw image of the embryonic zebrafish heart at 48 hpf with anatomical labels including the sinus venosus (SV) and the atrioventricular canal (AVC) B) The raw image with cardiac phase-based background subtraction used for μ PIV with labeled reference lines. C) Masks applied to the heart with background subtraction (median of all images) to define a region of fluid, shown in red and labeled as Blood Mask, and a region of the cardiac wall, shown in blue and labeled as the Wall Mask. D) Pressure integration paths from a single node to a fluid-solid boundary. Blue denotes paths for a node along the fluid-solid boundary and dotted red denotes a node in the flow domain.

Figure 2: Velocity profiles across the atrial inlet during atrial systole (top) and at the AVC during atrial diastole (bottom), as computed through μ PIV. Plots are mapped on a scale of 0-1.0T, corresponding to the complete cardiac cycle.

Figure 3: Relative pressure contours and velocity vectors at the onset of atrial systole, with the fraction of the cycle shown in the bottom right corner of each image. The pressure is normalized by the heart rate (HR) and viscosity (μ). A 0 pressure condition is set at the SV.

Figure 4: Relative pressure contours and velocity vectors during atrial systole, with the fraction of the cycle shown in the bottom right corner of each image. A 0 pressure condition is set at the lowest pressure of the ventricle.

Figure 5: Relative pressure contours and velocity vectors during atrial diastole, with the fraction of the cycle shown in the bottom right corner of each image. A 0 pressure condition is set at the SV.

364 Figure 6: A) Box plot, demonstrating variations in the shear rate over the cardiac cycle. The edge of each
365 box shows the 25th and 75th percentiles, with a line going across the median. + denotes the mean and
366 bars extend to maximum and minimum values. B) The pressure drop from the SV to the leading edge of
367 atrial expansion (Atrial Inlet) and the pressure drop across the AVC (AVC) for various viscosity models.
368 Black lines denote the pressure drop across the atrial inlet when it is open, while the gray lines denote
369 the pressure drop across the AVC, when it is open. C) Changes in viscosity relative to shear rate for
370 Newtonian and non-Newtonian viscosity models.
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Figure 1

Figure 2

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Figure 4

Figure 5

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