Original Contribution

40-MHZ ECHOCARDIOGRAPHY SCANNER FOR CARDIOVASCULAR ASSESSMENT OF MOUSE EMBRYOS

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(Received 27 April 1998; in final form 3 August 1998)

Abstract—Congenital heart disease results from genetic defects that are manifested at early stages of embryogenesis. The mouse is the preferred animal model for studies of mammalian embryonic development and for an increasing number of human disease models. A number of genes identified in the mouse are critical for normal cardiovascular development, but an understanding of the underlying mechanisms regulating heart development is still incomplete, in part because of the lack of methods to measure hemodynamics in live mouse embryos. We describe the development of a 40-MHz ultrasound scanner, which allows image-guided continuous-wave and pulsed Doppler blood flow measurements in mouse embryos, in utero, at the critical early developmental stages. Doppler waveforms acquired from mouse embryonic umbilical vessels, descending aorta, and cardiac ventricles are presented to demonstrate the utility of the method. By combining image-guided ultrasound Doppler with the many available mouse mutants, this approach should lead to new insights into embryonic cardiovascular structure–function relationships. © 1998 World Federation for Ultrasound in Medicine & Biology.

Key Words: 40-MHz echocardiography, Ultrasound imaging, Doppler ultrasound, Mouse embryo, Congenital heart disease.

INTRODUCTION

Congenital heart disease affects one in 100 children, making it the most common form of birth defect and the leading congenital cause of mortality in the first year of life. Echocardiographic imaging and Doppler in the frequency range 3–7.5 MHz have become established methods of assessing fetal cardiovascular structure and function (Snider and Serwer 1990), and transvaginal transducers have made clinical measurements possible as early as late first trimester (Wladimiroff et al. 1991). However, at this stage, the heart is almost completely formed, and monitoring of heart defects takes place well after the critical developmental stages. Indeed, functional development of the early embryonic cardiovascular system is not well understood. With the current trend towards in utero diagnosis and management of congenital heart disease, it is imperative to obtain a complete understanding of cardiovascular development at all stages of embryogenesis.

Extensive genetic information and transgenic techniques available in the mouse have led to its wide use in studies of mammalian development and models of human disease. In particular, a number of genes essential for normal cardiac development have been identified in the mouse through gene targeting (Rossant 1996). Although techniques have evolved rapidly to produce specific alterations in the genetic makeup of the mouse, technologies for the analysis of mutant phenotypes have not developed at the same pace. The heart is the first organ to develop, and the progression of normal embryogenesis depends on the early establishment of a functioning cardiovascular system. Mutations of genes suspected of being involved in cardiovascular development have been particularly difficult to analyze because of the lack of methods to measure blood flow and heart dynamics in mouse embryos (Chien 1996).

There is currently no high-resolution method available for noninvasive study of cardiovascular development in the mouse. Histology (Kaufman 1992), scanning
and back regions of the anesthetized pregnant mice were detected after overnight mating. The lower abdomen (E0.5) being defined as noon of the day a vaginal plug was provided to demonstrate the utility of this approach.

Examples of UBM images and Doppler waveforms from blood flow measurements in early stage mouse embryos. 40-MHz continuous-wave (CW) (Christopher et al. 1996) and pulsed Doppler systems (Christopher et al. 1997) Doppler signals. In either case, the basic Doppler principle is used to detect frequency shifts, which interfere with data acquisition. Embryos were staged in days of gestation, with day 0.5 (E0.5) being defined as noon of the day a vaginal plug was detected after overnight mating. The lower abdomen and back regions of the anesthetized pregnant mice were wet shaved, and a water bath was fitted on the skin as described previously (Srinivasan et al. 1998). Briefly, the mouse was laid in the lower level of a two-level stage and a plastic petri dish (100-mm diameter), modified by punching a 25-mm diameter hole in the center, was pinned over the mouse, exposing the shaved skin to the water bath. The 100-mm water bath provided adequate room for scanning the imaging transducer and positioning the Doppler transducer to measure blood flow in embryos lying below the skin surface (Fig. 1).

MATERIALS AND METHODS

Animals

All animals used in these studies were maintained according to protocols approved by the Institutional Animal Research and Care Committee at New York University Medical Center. Timed pregnant Swiss-Webster mice (Taconic, Germantown, NY, USA) were anesthetized with sodium pentobarbital (0.5 mg per 10 g body weight, injected intraperitoneally) mixed with magnesium (MgSO4 · 7H2O, 1 mg per 10 g body weight) as a mild muscle relaxant to decrease spontaneous uterine contractions, which interfere with data acquisition. Embryos were staged in days of gestation, with day 0.5 (E0.5) being defined as noon of the day a vaginal plug was detected after overnight mating. The lower abdomen and back regions of the anesthetized pregnant mice were

Ultrasound microcopy imaging system

The UBM system used in this study was a custom-built scanner, very similar to one previously described (Turnbull et al. 1995a). Differences between this scanner and the previous version include a new mechanical probe based on a linear induction motor (ML2-0806-005JBT, Northern Magnetics, Santa Clarita, CA, USA) coupled to an LVDT position encoder (LVDT 0242-0000, Transtek, Ellington, CT, USA), and a single 500-MHz, 8-bit analog-to-digital (A/D) converter (SPT 7750, Signal Processing Technologies, Colorado Springs, CO, USA) on the digitizing board. The front-end protection circuitry, monocyte pulse generator (AVB2-C-OCIC, Avtech ElectroSystems, Ottawa, Canada) and detector circuitry are identical to those described in the previous article (Turnbull et al. 1995a). UBM transducers are scanned linearly to produce 512 × 512 × 8 bit pixel images, covering an area that is variable from 2 mm × 2 mm to 8 mm × 8 mm, at frame rates up to 10 images per second. In this report, all images were 8 mm × 8 mm, acquired at a frame rate of 8 images per second. The linear induction motor was mounted on a motorized three-axis positioning stage (UMR8.25 micrometer stages with BM25cc actuators and MotionMaster 2000 servo controller, Newport-Klinger, Irvine, CA, USA). Fine XYZ positioning of the UBM image plane was maintained using a joystick controller (model MM2000-J, Newport-Klinger). All UBM images in this study were obtained using a 40-MHz, spherically focused polyvinylidene difluoride (PVDF) transducer fabricated as described previously (Turnbull et al. 1995a). The transducer aperture was 5 mm, with a focal length of 10 mm, resulting in measured lateral resolution (−6 dB) of 90 μm and axial resolution of 30 μm. The penetration depth, imaging mouse embryos with this transducer, was 7–10 mm.

Continuous-wave and pulsed Doppler systems

The UBM scanner has been augmented with 40- to 50-MHz Doppler transducers and circuitry to produce both CW (Christopher et al. 1996) and pulsed (Christopher et al. 1997) Doppler signals. In either case, the basic Doppler principle is used to detect frequency shifts,
which are simply related to the velocity of the blood flowing in the interrogating ultrasound beam of the Doppler transducer (Fig. 2a):

\[
f_d = 2 \cdot f_0 \cdot \cos \theta \cdot \cos(\phi/2) \cdot (v_b/c)
\]  

(1)

where \(f_d\) is the shifted frequency of the received signal, \(f_0\) is the frequency transmitted by the Doppler transducer, \(\theta\) is the angle between the flow direction and the Doppler ultrasound beam direction, \(\phi/2\) is the half angle between the transmit and receive beam directions for the CW transducers (\(\phi = 0\) for pulsed Doppler transducers), \(v_b\) is the velocity of the flowing blood, and \(c\) is the speed of sound in the medium. For phantom studies, the speed of sound in water was assumed to be 1500 m/s, whereas for \textit{in vivo} blood velocity measurements, we assumed a speed of sound in blood of 1580 m/s (Lockwood et al. 1991). Our implementation of the system electronics was the same as described previously for both the CW (Christopher et al. 1996) and pulsed (Christopher et al. 1997) Dop-
ler systems, using a quadrature oscillator (ADS-432-1176A, Sciteq Electronics, San Diego, CA, USA) as the frequency source, a broadband gated amplifier (model 310, Matec, Warwick, RI, USA) to gate the pulsed Doppler tone bursts, and a digital audio tape recorder (Panasonic SV-3700) to record and play back the in-phase (I) and quadrature (Q) Doppler signals.

CW Doppler transducers were fabricated from 80-μm thick wafers of 36° Y-cut lithium niobate (LiNbO$_3$, Crystal Technology, Palo Alto, CA, USA), cut into matched pairs of 1- to 1.5-mm diameter disks, and air back mounted in either SMA (Christopher et al. 1996) or SMB electrical connectors. These transducers then are housed in custom-machined plexiglass holders to align the overlapping transmit and receive Doppler beams (Figs. 2b and c). The CW transducers were designed with an angle (φ) of 30° between the transmit and receive ultrasound beams (Fig. 2a). Pulsed Doppler measurements were made with a focused PVDF transducer built into an SMA connector (Fig. 2d). All Doppler transducers fabricated for this study were operated at a center frequency between 43 and 51 MHz (Table 1). Axial and lateral beam widths of each of the Doppler transducers were measured by scanning the transducer over a 12.5-μm diameter glass target. The lateral beam widths reported in Table 1 are the average of two measurements taken in perpendicular lateral directions.

For in-line blood flow (θ = 0), eqn (1) can be reduced to give a simple relationship between the Doppler shift frequency and blood velocity:

$$f_d = K \cdot v_b$$

where $f_d$ is in Hz, $v_b$ is in mm/s, and the calibration factor $K$ is 52.57 mm$^{-1}$ for the 43-MHz CW Doppler system, and 62.36 mm$^{-1}$ for the 51-MHz pulsed Doppler system.
Ultrasound microscopy image-guided Doppler measurements

The Doppler transducers were mounted on a three-axis micromanipulator (model M-152; Narishige, Tokyo Japan), attached to the motorized motion stage housing the imaging transducer (Fig. 1b). The micromanipulator was used to align the Doppler beam in the UBM image plane and to calibrate the position of the Doppler sampling volume. The SMB CW and single SMA pulsed Doppler transducers were found to be easiest to position and use for UBM-guided Doppler measurements, because they were sufficiently small to allow simultaneous Doppler and UBM imaging, whereas the SMA CW Doppler transducers had to be repositioned with the micromanipulator for each Doppler measurement.

The position of the Doppler sample volume in the UBM image plane required calibration. First, the angle between the Doppler beam and the vertical direction of the UBM image plane was set to 45° (Fig. 1b) by pulsing the Doppler transducer and adjusting the angle to maximize the pulse-echo reflection from a 45° prism (D32,332 right-angle prism, Edmund Scientific, Barrington, NJ, USA). A spherical glass 150-μm diameter target was mounted in a small water tank at the center of the UBM image plane, determined by maximizing the UBM image brightness of the glass target. The Doppler transducer was pulsed while adjusting its position in three orthogonal directions until the amplitude of the return echo from the point target was maximized, ensuring that the Doppler sample volume was centered in the UBM image plane. For the CW Doppler transducers, the transmit element was pulsed while adjusting its position and the echo signal from the receive element was maximized, again to ensure that the Doppler signals were sampled from the same plane as the UBM image. Repeatability of this calibration procedure was well within the 500-μm precision of the micromanipulator position scale. The position of the Doppler sample volume in the UBM image plane was calibrated at the beginning of each experiment, and then checked again after blood flow measurements were obtained from mouse embryos.

Doppler signal processing

A Labview (National Instruments, Austin, TX, USA) virtual instrument was written to acquire and process the in-phase (I) and quadrature (Q) Doppler signals into a spectrogram (time-frequency) graphical output. The signals were digitized by an A/D board (AT-2150C, National Instruments) for a total time T with a variable sampling rate up to a maximum of 51.2 kHz (i.e., maximum Nyquist frequency = 25.6 kHz). Examination times for analyzing blood flow patterns were typically between 3 and 5 s, at sampling rates between 8 and 16 kHz. This rate was fast enough to sample all Doppler frequency shifts found in the mouse embryos studied (see Results). The nonstationary Doppler data were windowed with a Hanning filter to resolve, in time, fast changes in the flow of the circulating blood. The width of the window \( T_w \) was small compared to \( T \), being held constant at 256 sample points. For example, at a typical sample rate of 12 kHz, \( T_w = 21 \) ms. The complex windowed I and Q data, \( I + jQ \), were then Fourier-transformed to obtain the Doppler frequency shifts (Powars 1990). The magnitude spectrum was incorporated as one line in the resulting spectrogram (x-axis = time, y-axis = frequency shift or velocity, and amplitude is represented as 8-bit gray scale). This procedure was repeated sequentially for the entire time series, at each step translating the window an amount \( T_\Delta \), which was held constant at 50 sample points (for a 12-kHz sample rate, \( T_\Delta = 4.2 \) ms). Figure 3 shows an example illustrating generation of the Doppler spectrogram for a simple string phantom, rotating at a single fixed speed (see Results). The variations in velocity about the mean value (Fig. 3) represent the real movement of the string perpendicular to its main linear motion due to vibrations transferred from the motor/pulley assembly.

RESULTS AND DISCUSSION

 Calibration of the continuous-wave and pulsed Doppler systems

A string phantom was used to verify that the output of the CW and pulsed Doppler systems gave expected Doppler frequency shifts for known string velocities. A
A string of length 260 mm was stretched over two pulleys, with one of the pulleys attached to a variable speed DC motor. The string velocity was calculated by averaging the rotational period over 10 cycles and multiplying by the length of the string. The Doppler beam was aligned at 45° to the string (see Materials and Methods). Figure 3 shows the generation of a spectrogram for the string moving at a constant velocity of 30 mm/s, corresponding to a Doppler frequency shift close to 2 kHz. The existence of a spurious peak in the negative frequency range of the magnitude spectrum indicates approximately 30-dB directional isolation, due to imperfect frequency amplitude balancing of the I and Q channels (Christopher et al. 1997), which were matched to a tolerance of 5%.

Conversion between measured Doppler frequency shift and string velocity were obtained using the Doppler eqn (1), with $\theta = 45^\circ$, and assuming a speed of sound in water of 1500 m/s. For each string velocity, the spectrogram was generated and the mean frequency was calculated using a power weighted average. Figure 4 shows the plot of the mean Doppler frequency shift versus string velocity for both the CW and pulsed Doppler systems. The dashed line shows the linear regression fit of the data, whereas the solid line is the theoretical line as predicted by the Doppler equation. As demonstrated by these plots, both systems produce Doppler frequency shifts in excellent agreement with string velocity over the entire range tested. Finally, the direction of the string...
was reversed to check that the results were the same except that the spectrograms appeared as negative frequencies (data not shown).

In utero blood flow measurements from umbilical vessels

Umbilical blood vessels were easily identified from in utero UBM images, as they form the connection between the embryo and the maternal placenta (Figs. 5 and 6). In addition, the moving blood in the vessels is highly echogenic, resulting in the prominent appearance of the moving speckle pattern on real-time UBM images (Srinivasan et al. 1998). The CW Doppler system was most sensitive for measuring umbilical waveforms, but the sampling volume was large so that arterial and venous flow patterns were detected simultaneously (Fig. 5).

The high spatial resolution of the pulsed Doppler system...
(Table 1) was useful for obtaining separate blood flow waveforms from the umbilical artery and vein (Fig. 6). Using the UBM-guided pulsed Doppler system, blood flow waveforms were measured in the umbilical arteries of mouse embryos staged between E9.5 and E14.5 (Fig. 7). Our results indicate that the peak blood velocity in the umbilical artery increases from about 10 mm/s at E9.5 to 60 mm/s at E14.5 (Fig. 7). The apparent decrease in blood velocity between E10.5 and E11.5 (Fig. 7) reflects the normal variation in blood velocity between individual embryos and is not a generally observed phenomenon. In these measurements, the Doppler beam was
aligned as closely as possible to the assumed direction of
blood flow (Figs. 5 and 6) and eqn (2) was used to
convert Doppler shift frequencies to blood velocity val-
ues.

The ability to use UBM-guided Doppler to measure
umbilical blood flow in mouse embryos should be very
useful for characterizing functional defects in mutant
embryos with abnormal placental development. For ex-

Fig. 8. UBM-guided Doppler measurements in the mouse embryonic cardiovascular system. (a) In utero UBM image of an E14.5 mouse embryo with the pulsed Doppler sample volume (white rectangle) positioned over the descending aorta (DA). LV = left ventricle. Smallest scale increments (right) = 100 μm. (b) Pulsed Doppler waveform from the descending aorta (DA), with flow away from the transducer (negative). (c) In utero UBM image of an E12.5 embryo with the pulsed Doppler sample volume (white rectangle) positioned between the cardiac ventricle (V) and atrium (A). U = umbilical cord. Smallest scale increments (right) = 100 μm. (d) Pulsed Doppler measurement of ventricular inflow (negative) and outflow (positive) waveforms. (e) In utero UBM image of an E10.5 embryo with the CW Doppler sample volume (white box) positioned over the ventricle (V) and atrium (A), similar to (c). N = neural tube cavity. Scale bar = 1 mm. (f) CW Doppler measurements of ventricular inflow (negative) and outflow (positive) waveforms, where initial ventricular filling (E), followed by filling due to atrial contraction (A), can be identified on the inflow waveform.
ample, we previously demonstrated that mutant mouse embryos lacking the gene VCAM-1 can be identified on UBM images from early embryonic stages (Srinivasan et al. 1998). VCAM-1 mutants exhibit two distinct phenotypes (Gurtner et al. 1995; Kwee et al. 1995): half of the mutants fail to form a normal connection between the allantois (embryonic umbilical cord) and the chorion (maternal placenta), and subsequently die on or before E11.5. The other half of the VCAM-1 mutants have an apparently normal connection between allantois and chorion, but still die 1 d later (E12.5) from a primary defect caused by the loss of VCAM-1 (Kwee et al. 1995).

**Blood flow measurements from embryonic aorta and cardiac ventricles**

UBM image-guided Doppler measurements also were made in the developing heart and aorta of mouse embryos (Fig. 8). The moving speckle pattern of flowing blood on real-time UBM images made it possible to visualize large blood vessels, such as the aorta (Fig. 8a), great veins, and cerebral vessels inside developing embryos. UBM-guided Doppler measurements of the descending aorta showed blood flow patterns that primarily were unidirectional, with peak velocities up to 80 mm/s (Fig. 8b).

The beating cardiac chambers were identified easily on real-time UBM images (Figs. 8c and 8e). When the Doppler sample volume was positioned over the atrioventricular canal, the resulting Doppler waveforms were characteristic of previously described ventricular inflow–outflow patterns (Gui et al. 1996), with a sharp-peaked, high-velocity inflow waveform followed by a broad-peaked, lower velocity outflow waveform (Fig. 8d). Of particular interest was the observation of biphasic inflow waveforms as early as E10.5, demonstrating a low-velocity peak (E) resulting from initial ventricular filling, followed by a higher velocity peak (A) due to ventricular filling after atrial contraction (Fig. 8f). Previous investigations with 7.5-MHz Doppler revealed similar patterns, but only at later (E15.5) embryonic stages (Gui et al. 1996). Our results suggest that this biphasic ventricular inflow pattern is present from very early embryonic stages in the mouse and may have been missed in earlier studies because of insufficient resolution and/or improper alignment of the Doppler beam. Comparison of the inflow waveforms shown in Figs. 8d and 8f demonstrate that the biphasic pattern was easier to detect when the blood flow was in-line with the Doppler beam (Fig. 8f).

A systematic study of peak inflow and outflow velocities has not been performed at this time, but preliminary measurements show peak velocities lying between those measured in similar stage embryos measured *ex utero* with 20-MHz pulsed Doppler (Keller et al. 1996) and *in utero* with 7.5-MHz Doppler echocardiography (Gui et al. 1996). For example, peak inflow velocities were close to 120 mm/s at E10.5 (Fig. 8f), compared to 220 mm/s (Gui et al. 1996) and 60 mm/s (Keller et al. 1996). In these preliminary experiments, the maximum blood velocities measured in mouse embryos were approximately 150 mm/s, corresponding to Doppler shift frequencies of <10 kHz, even with the 50-MHz pulsed Doppler probe. As mentioned previously (see Materials and Methods), the Doppler systems implemented on our scanner can sample signals with Doppler shift frequencies up to 25 kHz.

**SUMMARY**

Image-guided 40- to 50-MHz CW and pulsed Doppler instrumentation has been implemented on a 40-MHz UBM system. The instrumentation was verified using a string phantom to demonstrate the validity of the measured Doppler shift frequencies/velocity values. Blood flow waveforms from umbilical vessels, aorta, and cardiac ventricles were measured in mouse embryos over a range of embryonic stages (E9.5–E14.5), which are approximately equivalent to 4–8 weeks of human development. These results demonstrate the feasibility of image-guided, noninvasive measurements of blood flow patterns in developing mouse embryos during the critical early stages of cardiovascular development.

The 40-MHz echocardiographic scanner described in this article allows noninvasive *in vivo* measurements of cardiac structure and function in mouse embryos. This should be of great value in the study of cardiovascular changes resulting from gene targeting experiments. Analysis of mutant phenotypes previously has been limited to static examinations of cardiac morphology in fixed specimens and functional measurements, which are either low resolution or invasive. The combination of high-resolution, *in vivo* structural and functional data available with 40-MHz echocardiography, and the increasing number of mutant mice showing cardiac defects, provides a new approach to studying the embryonic cardiovascular system. The study of normal and abnormal cardiovascular development should ultimately lead to new insights into embryonic cardiovascular structure–function relationships and provide new data relevant to understanding congenital heart disease.

**Acknowledgements**—This research was supported by a grant from the Whitaker Foundation. The original development of the Doppler systems used in this scanner was funded by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (NCIC). We thank Scott Baldwin for intellectual contributions.
and guidance during the development of this scanner, and Hong Liu and Brian Starkoski for technical support of this project. F. S. F. is a Terry Fox Scientist of the NCIC. D. H. T. is an Investigator of the American Heart Association, New York City Affiliate.

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