Correlation of Ventricular Area, Perimeter, and Conotruncal Diameter With Ventricular Mass and Function in the Chick Embryo From Stages 12 to 24

Bradley B. Keller, Norman Hu, and Edward B. Clark

Ventricular form and function are interrelated during cardiovascular development. The study of muscle mechanics requires the real-time measurement of length, area, or volume. Because volume measures are not currently possible in the embryonic heart, we tested the hypothesis that end-diastolic (ED) and end-systolic (ES) ventricular perimeter, area, and conotruncal diameter correlate with ventricular mass and function in the stage 12 to stage 24 white Leghorn chick embryo. Video images of the contracting heart were recorded at 60 Hz on 1/2" videotape and analyzed with a custom image-analysis workstation. ED and ES video fields were selected by maximum and minimum ventricular area and were planimetered for epicardial ventricular perimeter, area, and conotruncal diameter. Data are reported as (mean±SEM, n>8) and were tested by analysis of variance and regression analysis. Heart rate calculated from cycle length increased from 78±6 beats/min at stage 12 to 162±5 beats/min at stage 24. ED and ES area increased geometrically versus stage (y=0.53−0.08x+0.004x², r=0.96, p<0.001; and y=0.60−0.09x+0.004x², r=0.98, p<0.001, respectively). ED and ES perimeter and conotruncal diameter increased linearly versus stage (r=0.95, p<0.001; r=0.96, p<0.001; and r=0.93, p<0.001; r=0.93, p<0.001, respectively). Shortening fraction for each measurement increased from stage 12 to 16 or 18 then decreased. ED and ES perimeter, area, and conotruncal diameter also correlated with stroke volume, ventricular wet weight, and embryo and extraembryonic vascular bed wet weight (r>0.90 and p<0.001 for each measurement). Thus, epicardial ventricular measurements accurately reflect ventricular mass and function in the embryonic heart. (Circulation Research 1990;66:109-114)
Materials and Methods

We studied white Leghorn chick embryos at stage 12 (49 hours, n=12), stage 14 (53 hours, n=12), stage 16 (56 hours, n=11), stage 18 (67 hours, n=12), stage 21 (3.5 days, n=9), and stage 24 (4 days, n=8) of a 36-stage (21-day) incubation period. Visual landmarks including somite number, limb size, and cardiac morphology identify each stage. The stages selected each represent a doubling of embryonic wet weight. Fertile eggs were incubated blunt end up in a forced draft incubator at 38.5°C and constant humidity. When incubated blunt end up, the blastodisc floats to the top of the egg, and the embryo develops beneath the air cell. Access to the embryo was gained by opening the shell and incising a small region of the inner and outer shell membranes. The embryo was then positioned for imaging on a photomacroscopic stage.

Video images were acquired using a photomacroscopic (model M400, Wild), a Dage 70 series video camera (Dage-MTI, Michigan City, Indiana) with a grade 1 Newvicon tube, a fiberoptic light source (Dolan-Jenner Industries, Woburn, Massachusetts), a Magnavox PV9670 VHS video recorder, and a time-date generator (FORA model VTG-33). The Dage camera generated 60 sequential video fields per second in the interlaced mode. The image field was 1,000–2,000 μm in diameter, and the effective raster spacing was 4–8 μm. One pixel in the image equals 0.25% of the epicardial diameter. Real time ±0.005 seconds was recorded on each field. A 10-μm scale-scribed glass standard was recorded in the plane of each embryo after imaging.

Individual video fields were replayed by the video recorder and analyzed at a work station that included a minicomputer (Premium/286, AST Research, Irvine, California), a monitor (Multisync II, NEC Information Systems, Boxborough, Massachusetts), a frame-grabbing board (model M8, Targa), JAVA video analysis software and Microsoft mouse (Jandel Scientific, Corte Madera, California), and a Sony PVM1271Q multipurpose monitor.

The video recordings of each embryo were reviewed at 5 fields/sec (½2 the normal rate) to follow the pattern of blood flow from ventricle to conotruncus during the cardiac cycle. The measurement protocol for each embryo included 1) calibration of measurement software for length (in millimeters) and area (in square millimeters) with the scribed standard, 2) selection of maximum (end-diastolic [ED]) and minimum (ES) ventricular area images from three cardiac cycles, 3) planimetry of ventricular epicardial perimeter (in millimeters), ventricular area (in square millimeters), and external conotruncal diameter at the midconotruncal cushions (in millimeters) (Figure 1), 4) calculation of shortening fraction (s <i>ED</i>−<i>ES</i>)/<i>ED</i> for each variable, and 5) calculation of heart rate from the cycle length of consecutive ED images. Intraobserver and interobserver error of area measurement by planimetry of 10 pairs of distinct video fields was analyzed by <i>t</i> test and was not significant (<i>p</i> > 0.29 and <i>p</i> > 0.96, respectively).

The mean values for each variable at ED and ES at each stage were compared by analysis of variance and regression analysis across the stage range. Each variable was tested by linear and second-order regression analysis for its relation to stage-specific values for ventricular wet weight, embryo plus vascular bed wet weight, and stroke volume. Stroke volume was calculated from dorsal aortic flow velocity measured with a single-crystal 20-MHz pulsed-Doppler velocity meter and dorsal aortic diameter in separate experiments.

Results

Qualitative slow-motion video analysis of the contracting heart showed that at each stage the embryonic ventricle has a diastolic filling phase and a systolic ejection phase. Atrial contraction occurred during late diastole. The conotruncal cushions were separated at end diastole in all stage 12 to stage 18 embryos. Retrograde ventricular filling from the aortic sac occurred in all stage 12 embryos.

Heart rate increased from 78±6 beats/min at stage 12 to 162±5 beats/min at stage 24 (Table 1). Ventricular ED and ES area increased geometrically when expressed versus embryo stage, whereas perimeter and conotruncal diameter increased linearly (Figure 2). Ventricular ED and ES area increased linearly versus stroke volume across the stage range, while perimeter and conotruncal diameter increased asymptotically (Figure 3).

ED and ES ventricular area correlated linearly with ventricular wet weight and embryo plus vascular bed wet weight. ED and ES ventricular perimeter and conotruncal diameter increased asymptotically versus ventricular wet weight and embryo plus vascular bed weight (Tables 1–3).

The shortening fractions for area and perimeter increased from stages 12 to 16 then declined. The relation of conotruncal shortening to stage differed qualitatively and peaked at stage 18 (Figure 4).

Discussion

Accurate measurement of embryonic hemodynamic function is possible despite the technically challenging small size of the embryonic heart. We used a glass capillary servo-null pressure system and single crystal 20 MHz pulsed-Doppler meter to measure blood pressure and blood flow in the chick embryo during cardiac morphogenesis. Our study of epicardial ventricular measures across a 32-fold increase in ventricular weight tested the validity of these video measures as useful indexes of ventricular mass and function.

The atrioventricular and conotruncal cushions function as valves during the cardiac cycle to facilitate forward blood flow. We noted that the conotruncal cushions were separated by end diastole in stage 12 to stage 18 chick embryos and that retrograde filling of the ventricle from the conotruncus occurred only in stage 12 embryos. Forward flow through the conotrunc-
FIGURE 1. Representative images of a stage 21 chick embryo. Top panel: End diastole. Bottom panel: End systole. Epicardial ventricular border and midconotruncal diameter marked for planimetry. Standard is 1.0 mm. E, eye; L, limb bud; V, ventricle; DA, dorsal aorta. Arrow indicates conotruncus.
TABLE 1. Ventricular Measurements in Chick Embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>Heart rate (beats/min)</th>
<th>ED perimeter (mm)</th>
<th>ES perimeter (mm)</th>
<th>ED area (mm²)</th>
<th>ES area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>12</td>
<td>72±6</td>
<td>1.80±0.03</td>
<td>1.47±0.04</td>
<td>0.23±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>116±5</td>
<td>2.20±0.08</td>
<td>1.77±0.06</td>
<td>0.34±0.03</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>16</td>
<td>11</td>
<td>120±4</td>
<td>2.51±0.04</td>
<td>1.94±0.05</td>
<td>0.45±0.02</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>131±4</td>
<td>2.79±0.07</td>
<td>2.31±0.05</td>
<td>0.57±0.03</td>
<td>0.39±0.02</td>
</tr>
<tr>
<td>21</td>
<td>9</td>
<td>148±3</td>
<td>3.43±0.07</td>
<td>3.02±0.05</td>
<td>0.86±0.04</td>
<td>0.67±0.02</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>162±5</td>
<td>4.18±0.13</td>
<td>3.61±0.06</td>
<td>1.26±0.07</td>
<td>0.94±0.03</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ED, end-diastolic; ES, end-systolic.

**FIGURE 2.** Graphs showing two-dimensional measures from stages 12 to 24. Top panel: End-diastolic and end-systolic ventricular area versus stage. Middle panel: End-diastolic and end-systolic ventricular perimeter versus stage. Bottom panel: End-diastolic and end-systolic conotruncal diameter versus stage. All regression curves were fitted to raw data; plotted values are mean±SEM.

**FIGURE 3.** Graphs showing two-dimensional measures versus stage-specific values of stroke volume. Top panel: End-diastolic and end-systolic ventricular area versus stroke volume. Middle panel: End-diastolic and end-systolic ventricular perimeter versus stroke volume. Bottom panel: End-diastolic and end-systolic conotruncal diameter versus stroke volume. All regression curves were fitted to raw data; plotted values are mean±SEM.
cal cushions occurred only after the onset of active ventricular contraction. These observations suggest that ventricular-vascular coupling is present early in cardiac development and that coupling is maintained despite rapid changes in ventricular and vascular resistance and compliance during cardiac morphogenesis. The effects of acute and chronic altered loading conditions on ventricular-vascular coupling and subsequent cardiac morphogenesis is unknown.

Geometric changes in the developing heart have been studied by various techniques. Cinephotography was used to study the onset of contraction and the morphological development of the chick heart. Investigators used planimetry of film images to calculate ventricular volumes and cardiac outputs in normal chick embryos. Photograph has also allowed the measurement of embryonic ventricular function after acute volume loading during hypoxia, and after chronic treatment with ethanol.

However, there are significant differences in the ventricular measurements reported in these previous studies. Hughes reported phasic volume curves for 2.5–12-day embryos and found that ED ventricular volume increases from 0.8 to 60 mm³. Faber et al calculated phasic volume tracings for stage 16 to stage 25 embryos and showed that estimated stroke volume increases from approximately 0.04 to 0.65 mm³. Ruckman et al studied stage 11 to stage 15 embryos and reported stroke volumes of 0.007–0.152 mm³.

The use of formulas derived from models of solid geometry to process two-dimensional measurements of the embryonic heart has complicated the interpretation of these experimental results. Ventricular volume has been estimated by Simpson’s rule for a prolate ellipse and a prolate spheroid. However, the progressive remodeling of the embryonic heart during cardiac morphogenesis precludes the use of simple geometric estimates of ventricular volume, and there is currently no experimental evidence to support their application to the chick embryo.

Therefore, we reported the absolute values of perimeter, area, and conotruncal diameter and made no attempt to estimate ventricular volumes from these measures. We calculated the correlation of each epicardial measure to values for stroke volume measured by pulsed Doppler in separate experiments. Stroke volume measured by pulsed Doppler increases geometrically during cardiac development and reflects increasing ventricular performance. The high correlation of each epicardial measure with pulsed-Doppler–measured stroke volume supports the sensitivity of epicardial measures to changing ventricular function. Our results validate two-dimensional measures of the embryonic heart versus an independent measure of cardiac function.

The ventricular border selected for planimetry has also varied among investigators. Hughes traced the endocardial border for embryos incubated up to 4 days and traced the epicardial border in older embryos. Faber et al traced only the epicardial border, and Ruckman et al traced only the endocardial border. After stage 16, the smooth-walled endocardium is gradually replaced by a complex trabecular surface. Scanning electron micrographs show the trabecular ventricular wall to be much thinner than suggested by light microscopic images. Therefore, we selected the epicardial border for planimetry as the only consistent, traceable ventricular edge available across a broad stage range of cardiac development. One limitation of the use of epicardial measures of cardiac size is that the ratio of cavity space to myocardial area changes during the cardiac cycle. However, both ED and ES area measures are highly correlated with cardiac development. Therefore, we are in the process of quantifying ventricular wall topology from scanning electron micrographs, and this data will be used to refine our two-dimensional real-time measures.

### Table 2. Conotruncal Measurements in Chick Embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>ED diameter (mm)</th>
<th>ES diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>12</td>
<td>0.29±0.01</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>0.35±0.01</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>16</td>
<td>11</td>
<td>0.42±0.01</td>
<td>0.31±0.01</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>0.50±0.01</td>
<td>0.36±0.01</td>
</tr>
<tr>
<td>21</td>
<td>9</td>
<td>0.58±0.01</td>
<td>0.46±0.01</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>0.59±0.03</td>
<td>0.52±0.03</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ED, end-diastolic; ES, end-systolic.

### Table 3. Reference Values for Stage-Specific Wet Weights in Chick Embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Ventricular wet weight (mg)</th>
<th>Embryo and vascular bed wet weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.10±0.01 (15)</td>
<td>27.7±1.56 (20)</td>
</tr>
<tr>
<td>14</td>
<td>0.12±0.01 (10)</td>
<td>36.9±0.79 (20)</td>
</tr>
<tr>
<td>16</td>
<td>0.23±0.01 (10)</td>
<td>53.5±1.75 (17)</td>
</tr>
<tr>
<td>18</td>
<td>0.35±0.01 (10)</td>
<td>134.0±6.94 (16)</td>
</tr>
<tr>
<td>21</td>
<td>0.42±0.08 (10)</td>
<td>163.0±8.51 (16)</td>
</tr>
<tr>
<td>24</td>
<td>0.97±0.02 (10)</td>
<td>291.0±10.90 (16)</td>
</tr>
</tbody>
</table>

Values are mean±SEM. The number of chick embryos is indicated in parentheses.

**Figure 4.** Graph showing shortening fraction for ventricular area, perimeter, and conotruncal diameter from stages 12 to 24.
The frame rate of image acquisition significantly influences the accuracy of selecting true ED and ES images. Our imaging system acquires and displays consecutive individual video fields for a field rate of 60 Hz and provides 30 images/cycle at a heart rate of 120 beats/min. Frame rates of 16 Hz\(^2\) and 18 Hz\(^2\) result in a large percentage of nonimaged time during the cardiac cycle and a small number of frames around the times of ED and ES. For example, in a stage 16 embryo (heart rate of 120 beats/min), a frame rate of 18 Hz provides only 9 images/cardiac cycle and a single image around the time of ES or ED. Better temporal resolution is essential for the use of image analysis in hemodynamic and morphometric measures.

Differences in the techniques of embryo preparation before imaging may also explain discrepancies in experimental results. We imaged embryos in vivo, and heart rate for stage 12 to stage 24 embryos was similar to those reported for normal embryos; this finding indicates that embryos were recorded under physiological conditions.\(^5\) In four studies, embryos were removed from the shell before imaging.\(^5\),\(^7\),\(^12\),\(^13\) The effect of embryo relocation on cardiac function is unknown. We do know that the poikilothermic chick embryo responds rapidly to altered environmental temperature by altering heart rate in a reproducible manner.\(^16\),\(^17\) Increases in heart rate by as little as 25% adversely affect cardiac function.\(^18\) Heart rate was not reported in one study,\(^5\) and the wide variation in heart rate reported in another suggests that observations were made under nonphysiological conditions.\(^13\)

Conotruncal diameter correlated with embryo stage, ventricular wet weight, and stroke volume. Because the conotruncus contributes mechanically to cardiac output early in cardiac development\(^9\) but then regresses,\(^20\) the interpretation of these results is unclear. Developmental alterations in ventricular geometry, function, and vascular properties are additional factors that likely affect phasic conotruncal expansion and relaxation; thus, conotruncal diameter is a poor index of ventricular function in the chick embryo.

The direct correlation of epicardial ventricular area and perimeter with stage, ventricular wet weight, and embryo plus vascular bed wet weight supports the validity of these measures during rapid cardiac morphogenesis. Ventricular wet weight increases geometrically during cardiac development, and embryo plus vascular bed wet weight reflects hemodynamic demand.\(^2\)\(^9\) The increase in perimeter and area shortening fraction from stages 12 to 16 coincides with the decrease in vascular resistance that occurs during vascular bed expansion and may reflect an improvement in ventricular-vascular coupling. The decrease in shortening fraction noted after stage 16 coincides temporally with the timing of ventricular trabeculation and may reflect decreasing ventricular compliance. The validation of ventricular epicardial measures as representative of ventricular mass and function supports their use in the analysis of simultaneous measurements of physiology and morphology in the embryonic heart.

## References


**Key Words**: cardiovascular development • cardiac morphogenesis • ventricular function • chick embryo
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