Effect of increased pressure on ventricular growth in stage 21 chick embryos

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Division of Pediatric Cardiology, Department of Pediatrics, Department of Anatomy, and The Cardiovascular Center, University of Iowa Hospitals and Clinics, Iowa City, Iowa 52242; and Dorothy S. and Frederick W. Cook Pediatric Cardiology Research Laboratory, Division of Cardiology, Department of Pediatrics, University of Rochester School of Medicine, Rochester, New York 14642

Clark, Edward B., Norman Hu, Peter Frommelt, Gregg K. Vandekieft, James L. Dummett, and Robert J. Tomanek. Effect of increased pressure on ventricular growth in stage 21 chick embryos. Am. J. Physiol. 257 (Heart Circ. Physiol. 26): H55-H61, 1989.—We studied the effect of increased ventricular pressure on heart growth in the stage 21 (3.5-day) chick embryo. Ventricular pressure was increased by constricting the conotruncus with a loop of 10-0 nylon tied in an overhand knot. The embryos were reincubated, and physiology and cellular morphology were evaluated at successive stages of development, stages 21, 24, 27, and 29. Ventricular pressure was measured with a servo-null pressure system, and cardiac output was measured with a 20-MHz pulsed Doppler velocity meter. Ventricular and embryo dry weights were measured on an electronic microbalance, myocyte organelle composition was measured by a point counting technique, and cell growth response was measured by DNA and protein assay. The conotruncal loop increased ventricular pressure in experimental compared with control embryos, i.e., at stage 24, 2.88 ± 0.13 vs. 1.96 ± 0.05 (SE) mmHg (P < 0.05), respectively, without affecting cardiac output. Ventricular dry weight increased in experimental vs. control embryos, i.e., at stage 24, 114 ± 7 vs. 85 ± 1 µg (P < 0.05), respectively, whereas embryo weights were similar between the two groups. The difference in ventricular weights was due to myocyte hyperplasia, since organelle proportion of myofibrils and mitochondria, DNA-to-protein ratio, and myocyte area were similar in experimental voice control embryos. Thus the adjustment of myocardial mass to ventricular work occurs even during the earliest stages of embryonic development. Cardiac growth and morphogenesis are parallel but separable processes.

Heart size increases as a response to increased functional load. The mature animal's heart compensates for an increased work load by myocyte hypertrophy (4). In the immature animal, cardiac compensation includes both hypertrophy and hyperplasia of the cardiac myocytes (21). Little is known, however, about the response of the embryonic heart to increased functional demands. During the embryonic period, the heart grows rapidly as it develops from a muscle-wrapped tube to a septated four-chambered organ. Heart growth and morphogenesis are controlled in part by genetic information. However, the heart is functioning during development, pumping blood to the embryo and to extraembryonic circulation (7, 8). Thus extrinsic factors could affect the growth and morphogenesis of the cardiovascular system.

We hypothesized that an increase in cardiac work load during the embryonic period increases ventricular growth. We increased ventricular pressure by constricting the outflow tract of the embryonic heart with a loop of 10-0 nylon suture. Myocyte hyperplasia accounted for the increase in ventricular dry weight in experimental compared with control hearts.

Methods

Fertilized White Leghorn chicken eggs were incubated blunt end up to Hamburger-Hamilton (12) stage 21, 3.5 days of a 21-day incubation period. We reached the embryo by making a hole in the shell and incising the inner shell membrane. The outflow tract of the heart was constricted by tying a loop of 10-0 nylon suture at the midpoint of the conotruncus. Sham-operated embryos had a suture placed but not tied. Because of the rapid embryo growth, untied nylon fibers quickly became embedded in the conotruncal region destroying the outflow tract. Therefore, normal embryos served as controls. After each operation, the shell hole was sealed with paraffin film, and the egg was returned to the incubator.

We made the following physiological and morphological measurements in experimental and control embryos at stage 21 (3.5 days), 24 (4 days), 27 (5 days), and 29 (6 days).

Hemodynamic measures. For in ovo physiological measurements an egg was positioned on a dissecting microscope stage. Ventricular blood pressure was measured with a servo-null pressure system (WP Instruments) that is accurate over the range of 0-30 mmHg, that is accurate to ±0.10 mmHg, and that has a frequency response that is flat to 7 Hz. Rate of pressure development was electronically derived from the pressure curve by a differentiating channel (8). A 5- to 7-µm tip glass cannula was inserted through the ventricular wall. Zero pressure was determined by positioning the cannula tip in the extraembryonic fluid at the level of the ventricle. We averaged measurements from 40 consecutive pressure
cycles for each individual measurement.

Mean dorsal aortic blood flow, an index of cardiac output, was calculated from measured blood velocity and aortic diameter. We used a 20-MHz pulsed Doppler velocity meter to measure mean dorsal aortic blood velocity. This system is accurate over a range of 0–16 mm/s and accurate to ±0.50 mm/s (7). A 750-μm piezoelectric crystal was positioned at a 45° angle to the dorsal aorta at the level of the sinus venosus. The aortic diameter was measured with a filar micrometer eyepiece accurate to ±10 μm. Heart rate was determined by counting phasic pulsations between time lines.

**Embryo and ventricular weight.** Embryo and ventricular dry weight were measured on an electronic balance accurate to 10 μg. The embryo was removed from the shell and placed on a tared glass cover slip. A ventricle was weighed after removing the atria and great vessels. The tissue was dried in a 80°C oven for 3 h, transferred to a desiccator containing silica gel, and left for 12–24 h. Individual embryos were weighed, whereas 10 ventricles were pooled for each measurement of heart weight.

**Cardiac morphology.** For the analysis of morphogenesis, the hearts of experimental embryos were compared with those of stage-matched controls. Specifically, we determined the degree of atrial septation, atrioventricular septation, muscular ventricular septal formation, conotruncal septation, and the division of the conotruncus into the aorta and pulmonary artery. We analyzed each heart after excision from the embryo and before weighing.

**Myocyte proportion measurements.** Fractional myocyte cell volume was determined for mitochondria, myofibrils, glycogen, and sarcoplasm. We harvested a minimum of five ventricles at each stage, which were immediately fixed in isotonic 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, postfixed in 1% osmium tetroxide, and embedded in Spurr’s plastic.

Random thin sections were stained with uranyl acetate and lead citrate and photographed on a Hitachi H 600 transmission electron microscope. Each micrograph was printed at a magnification of 24,000.

We used a point-counting technique to analyze the micrographs after excluding cell vacuoles and nuclei. Ten micrographs from each of five embryos at each stage were counted through an overlay grid of 1,092 intercepts/micrograph. The number of points falling on a given structure (P) and a total number of points (P_total) is related to the relative volume fraction of the structure (V_i) per total cell volume (V_cell) as follows

\[ \frac{V_i}{V_{cell}} = \frac{P_i}{P_{cell}} \]

We determined the relative volume fraction for mitochondria, myofibrils, and glycogen. All other cytoplasmic structures including ground substance and lipid were categorized as sarcoplasm.

Intra- and interobserver errors were calculated from repeat counts of 10 micrographs by two observers and subsequently analyzed by t test. Intraobserver correlations were P > 0.25, and interobserver correlation was P = 0.62.

**Myocyte size.** We estimated myocyte size by measuring whole cell area from the random thin section electron micrographs printed at a magnification of 12,800. The cell margins of myocytes at stages 24 and 29 were traced with a standard planimeter and the area calculated. The individual making these measurements was unaware whether the micrographs were from experimental or control hearts.

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**FIG. 1.** Recording of ventricular phasic pressure and rate of pressure development (dP/dt) from a control and conotruncal loop stage 24 chick embryo. Note peak ventricular pressure curve recorded from experimental embryo.
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### TABLE 1. Hemodynamic and morphological measurements in conotruncal loop and control embryos

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Stage 21</th>
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<th>Stage 24</th>
<th>n</th>
<th>Stage 27</th>
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<th>Stage 29</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
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<tr>
<td>Ctl</td>
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<td>161±4</td>
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<tr>
<td>Con</td>
<td>20</td>
<td>153±5</td>
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<td>170±6</td>
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<td>186±9</td>
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<td>Ventricular peak systolic pressure, mmHg</td>
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<tr>
<td>Ctl</td>
<td>20</td>
<td>2.18±0.08</td>
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<td>Ventricular end-diastolic pressure, mmHg</td>
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<tr>
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<td>Cardiac output, mm³/s</td>
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<td>Ctl</td>
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<td>30</td>
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<td>Embryo weight, dry, mg</td>
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<tr>
<td>Ctl</td>
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<td>1.46±0.10</td>
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Values are means ± SE; n, no. of embryos. Ctl, conotruncal loop; Con, control. *P < 0.05.

The growth of the embryo was not altered by the conotruncal loop. From stage 21 to stage 29, dry embryo weight increased -10-fold. There was no difference between the dry weights of experimental conotruncal loop embryos and the controls (Table 1). Banding of the conotruncus did not adversely affect embryo growth.

The growth of the ventricle was related to ventricular systolic blood pressure. Ventricular weight was greater in experimental compared with control embryos (Fig. 2). The peak systolic pressure difference between the study groups was 0.40 mmHg at stage 21 and was sustained through stage 29, at which time the difference was 1.09 mmHg. End-diastolic pressure difference was 0.23 at stage 21 and 0.16 at stage 29 (Table 1). Although the differences in systolic and diastolic pressure between the banded and control hearts were very small, they are probably significant considering the low levels of pressure in the heart at these stages of development.

Other measures of cardiac function remained constant between the study groups (Table 1). Heart rate increased across the studied stages. Heart rate increased 20% between stages 21 and 29, but the increase was similar in experimental and control embryos. Thus the increase in ventricular pressure did not affect heart rate. Cardiac output was similar in the experimental and control embryos. The banding of the conotruncus did not affect the forward flow of blood from the embryonic heart.

The growth of the embryo was not altered by the conotruncal loop. From stage 21 to stage 29, dry embryo weight increased ~10-fold. There was no difference between the dry weights of experimental conotruncal loop embryos and the controls (Table 1). Banding of the conotruncus did not adversely affect embryo growth.

The growth of the ventricle was related to ventricular systolic blood pressure. Ventricular weight was greater in experimental compared with control embryos at stages 24, 27, and 29 (Fig. 2). The time between stages 21 and 24 is, on average, 12 h. Thus the growth stimulus within hours after placement of the band resulted in the increase in ventricular mass in excess of that formed during normal development (Fig. 3). At stage 29, the ventricle of an experimental embryo weighed, on average, 40% more than a ventricle from a control embryo.

Although there was an increase in ventricular mass in the experimental embryos, the morphological development of the experimental and control hearts was similar. For example, the septation of the atrioventricular canal, formation of the ventricular and atrial septa, and division of the conotruncus proceeded at the same rate. The only difference was the abnormal position length of the conotruncus in the experimental hearts similar to our previous observation (9).
Our measurements indicate that the increase of ventricular mass was due to myocyte hyperplasia based on the proportion of subcellular organelles, cell area, and DNA-to-total protein ratio. The relative proportion of subcellular organelles and the size of myocytes in the experimental and control hearts were similar (Figs. 4 and 5). The proportion of mitochondria was slightly increased in myocytes from experimental embryos at stages 21 and 27. The magnitude of the difference was <3% and within the range of biological variability. The proportion of myofibrils was also variable. At stages 24 and 29, the myofibrils were 3 and 4% greater in experimental than controls, respectively. At stages 24 and 29, the myocyte area in experimental and control embryos was similar. This measure of cell area taken from random orientation of myocytes indicates that there was no difference in myocyte size between the experimental and control hearts. The amount of total protein and DNA was increased in ventricles from experimental embryos at stages 27 and 29. However, the ratio of DNA to total protein was similar (Table 2).

DISCUSSION

The adjustment of myocardial mass to sustained functional load is a fundamental characteristic of the vertebrate heart. The type of cellular response depends on the maturity of the animal. In the adult heart, myocytes hypertrophy, accumulating additional contractile units and mitochondria with little myocyte replication (13). In the juvenile heart, myocytes undergo both hypertrophy and hyperplasia (20). In the fetal lamb heart, the response is primarily hyperplasia (11). The developmental aspects of this response to functional load are unclear.

We hypothesized that the mechanisms controlling heart mass function, even in the early embryo, serve to match heart size to functional demands during embryonic growth. These experiments show that this adaptive response does occur early in development during the period of organogenesis of the heart. At stage 21, there is little connective tissue in the heart. Therefore, myocardial mass increased in response to an increase in ventricular pressure.

The cellular growth response is likely hyperplasia rather than hypertrophy, based on two pieces of information. First, myocyte size and organelle proportion were similar between the hearts with conotruncal loop and the controls. Second, there was an increase in total protein and DNA in the ventricles banded with a conotruncal loop compared with controls consistent with growth. However, the similar ratio of DNA to total protein is more compatible with hyperplasia than with hypertrophy. With hypertrophic response, we expect the ratio of DNA to total protein to be less in the experimental hearts than in the controls. We are, however, unable to differentiate between the response in the myocyte and endocardial cell populations.

We chose to compare the mass of control and experimental hearts as an index of heart growth in the chick embryo. In the mature heart, investigators often measure wall thickness. This approach, however, is not practical in the embryo because the ventricular chamber is a spongeliike trabecular structure. The arrangement of trabecular struts precludes definition of the endocardial margins of the ventricular wall.

At the time of organogenesis, the heart is growing...
rapidly, which is evidenced by a doubling of heart and embryo weight between the stages we studied. Yet, the morphological complexity of the experimental and control hearts was similar. Thus additional work load on the embryonic heart increased the rate of heart growth without changing the rate of embryo growth or morphogenesis of the ventricle.

Conotruncal constriction affected the formation of the posterior conus, resulting in an increase in mitral aortic separation and interfered with the normal assignment of the aorta to the left ventricle (9). This is likely a mechanical deformation produced by the nylon loop and unrelated to ventricular growth.

The increase in ventricular mass is the end stage of a complex process. Other compensatory responses may precede the increase in ventricular mass. The rapid adjustment of ventricular pressure to conotruncal constriction suggests that the primitive heart is not pumping at the peak of its function curve. Functional reserve is present in the neonatal and mature rat heart (10) and is also likely present in the embryonic heart.

During organogenesis, sarcomeres are assembled within the sarcoplasm. Initially, the myoblast cannot be differentiated from other mesenchymal cells (16). With the formation of bilateral cardiac tubes, contractile elements appear and begin to contract, heralding the initiation of circulatory function. The earliest stages of myocyte function occur before imposition of hemodynamic load and are independent of physiological function. However, at stage 21, the heart is functioning to pump blood to the embryo (7).

An increase in work load may affect the number of functioning contractile units within a myocyte. The myocytes are still in the process of producing and assembling contractile units. In our previous study of normal chick myocytes (8), the percent of myofibrils increased slightly over these stages, but there was no increase in proportion in mitochondria. Although there was no difference in the absolute number of myofibrils, the number of functioning myofibrils may be different between growth-accelerated and control hearts. At present, we cannot differentiate between myofibrils that are fully assembled, but not yet capable of coordinated contraction, and those that are fully functional.

The increase in ventricular mass occurred before the onset of functional autonomic innervation. In the mature heart an increase in autonomic tone has been implicated in the increase in cardiac mass (1). Yet, the mechanism involved in the early chick embryo is probably independent of autonomic innervation. The stage 21 heart is preinnervated. The parasympathetic limb is functional at stage 39, day 12, and the sympathetic limb is functional at stage 42, day 17 (17, 19). Adrenergic receptors are present much earlier in development. β-Receptors are present in the stage 21 ventricular myocyte and in the stage 24 extraembryonic vascular bed (6, 19). Thus it is unlikely that adrenergic stimulation is a primary mechanism for accelerated cardiac growth in the chick embryo.

In other biological systems, cell division and growth are under control of tissue-level growth factors and antiproliferative factors (17). Studies in the spontaneously hypertensive rat suggest that mRNA for platelet-derived growth factor-β (PDGF-β) is greater than in controls (22). The interrelationship of peptide hormones and the adjustment of myocardial mass is only now becoming apparent. We speculate that a growth factor(s) produced within the developing ventricle regulate myocyte growth during ventricular morphogenesis.

This study also influences how we think about cardiac development. Cardiac morphogenesis and growth are often considered inseparable. This experiment suggests that heart growth adjusts to environmental factors without altering the pattern of morphogenesis. We do not know, however, whether the increase in heart mass was associated with an alteration in the fabric of the myocardium, which is the way the myofibrils are arranged in the complex pattern of the ventricle walls.

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REFERENCES


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