

Ventricular diastolic filling characteristics in stage-24 chick embryos after extra-embryonic venous obstruction

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Summary

Alteration of extra-embryonic venous blood flow in stage-17 chick embryos results in well-defined cardiovascular malformations. We hypothesize that the decreased dorsal aortic blood volume flow observed after venous obstruction results in altered ventricular diastolic function in stage-24 chick embryos. A microclip was placed at the right lateral vitelline vein in a stage-17 (52–64 h of incubation) chick embryo. At stage 24 (4.5 days of incubation), we measured simultaneously dorsal aortic and atrioventricular blood flow velocities with a 20-MHz pulsed-Doppler velocity meter. The fraction of passive and active filling was integrated and multiplied by dorsal aortic blood flow to obtain the relative passive and active ventricular filling volumes. Data were summarized as means \pm S.E.M. and analyzed by *t*-test. At similar cycle lengths ranging from 557 ms to

635 ms ($P>0.60$), dorsal aortic blood flow and stroke volume measured in the dorsal aorta were similar in stage-24 clipped and normal embryos. Passive filling volume (0.07 ± 0.01 mm³) was decreased, and active filling volume (0.40 ± 0.02 mm³) was increased in the clipped embryo when compared with the normal embryo (0.15 ± 0.01 mm³, 0.30 ± 0.01 mm³, respectively) ($P<0.003$). In the clipped embryos, the passive/active ratio was decreased compared with that in normal embryos ($P<0.001$). Ventricular filling components changed after partially obstructing the extra-embryonic venous circulation. These results suggest that material properties of the embryonic ventricle are modified after temporarily reduced hemodynamic load.

Key words: chick embryo, atrioventricular function, blood flow velocity, venous clip model.

Introduction

The formation of the four-chambered heart is a complex and dynamic interaction between the basic gene program that regulates growth and differentiation and the mechanical forces generated by the functioning heart. To study the influence of hemodynamics on heart morphogenesis, we used a chick embryo intervention model in which extra-embryonic blood flow can be manipulated. Intracardiac blood flow patterns were altered by a redirection of venous inflow through permanent obstruction of the right vitelline vein with a microclip. We previously showed that this intervention produced a specific set of cardiac malformations, namely ventricular septal defects, valve anomalies and pharyngeal arch artery malformations (Hogers et al., 1995, 1997). The similarity of these structural cardiac anomalies in the chick embryo to those observed in patients with congenital heart disease argues for the importance of intracardiac hemodynamics as a key epigenetic factor in embryonic cardiogenesis (Hove et al., 2003).

We recently demonstrated that venous clipping has major acute effects on hemodynamics in the stage-17 chick embryo

(Stekelenburg-de Vos et al., 2003). For the total study period of 5 h, dorsal aortic blood flow remains lower than baseline values, whereas heart rate shows a recovery to baseline within 2 h of clipping (Stekelenburg-de Vos et al., 2003). This dramatic reduction in total circulating blood volume after clipping could influence the functional characteristics of the embryonic heart. Since the early developing cardiovascular system is not yet innervated (Pappano, 1977), cardiovascular function is sensitive to mechanisms or interventions that alter hemodynamic load, such as in the venous clip model. In contrast to the mature cardiovascular system, the embryonic heart lacks the ability to acutely alter heart rate to compensate for reduced ventricular preload (Casillas et al., 1994). We therefore hypothesize that the decreased blood volume flow observed in the venous clip embryo would affect ventricular diastolic function.

Embryonic diastolic function can be investigated using pulsed-Doppler measurements of blood velocity and flow. The combination of simultaneous atrioventricular and dorsal aortic

blood flow profiles accurately defines passive and active ventricular filling volumes (Hu et al., 1991). We studied embryonic cardiovascular performance in the stage-24 chick embryo during normal growth and development and after venous clip intervention.

Materials and methods

Fertilised White Leghorn chicken (*Gallus domesticus* L.) eggs (Charles River Laboratories, Extertal, Germany) were incubated blunt-end up at 37–38°C and staged according to Hamburger and Hamilton (1951). Embryos that were dysmorphic or exhibited arrhythmias or overt bleeding were excluded. At stage HH 17 (52–64 h of incubation), the embryo was exposed by creating a window in the shell followed by removal of the overlying shell membranes. Adjacent to the right lateral vitelline vein, the vitelline membrane was removed and a small incision was made in the yolk sac membrane. An aluminum microclip was used to clip the right lateral vitelline vein (Hogers et al., 1995, 1997). After venous ligation, the window was sealed with tape and the eggs returned to the incubator until stage HH 24 (4.5 days of incubation).

Dorsal aortic blood flow velocity and atrioventricular (AV) blood flow velocity were recorded using a 20-MHz pulsed-Doppler velocity meter (model 545C-4; Iowa Doppler Products, Iowa City, IA, USA). Dorsal aortic blood velocity was measured with a 750- μm piezoelectric crystal positioned at a 45° angle towards the dorsal aorta at the level of the developing wing bud. Internal aortic diameter was calculated from a magnified video image displaying the dorsal aorta using a custom-built analysis program (IMAQ Vision; National Instruments, Austin, TX, USA) (Ursem et al., 2001). Atrioventricular blood flow velocity was measured with a second crystal positioned at the apex of the heart towards the AV orifice. The Doppler audio signals were digitized at 24 kHz and stored on hard disk. Using complex fast Fourier transform analysis, the maximum velocity waveform was reconstructed. A more detailed description of this method has been published previously (Ursem et al., 2001). Passive filling (P) was defined in the AV flow velocity waveform from end-systole to the onset of the A-wave, and active filling (A) from the onset of the A-wave to the onset of systole (Fig. 1). Portions of passive and active filling overlapped each other at faster heart rates. The demarcation between the passive and active velocities was dependent on heart rate but was most conspicuous as heart rate slowed. Therefore, heart rate was decreased to 100 beats min^{-1} by cooling of the embryo. Although environmental temperature directly influences hemodynamics (Wispe et al., 1983), a slowed heart rate of approximately 100 beats min^{-1} was necessary to discriminate between the passive and active filling phase and to study both groups under similar conditions. Cycle length was defined as the time between consecutive beats obtained from the dorsal aortic velocity waveform. Dorsal aortic and both passive

and active AV velocity profiles were integrated over time. Dorsal aortic blood flow, an estimate of cardiac output, was calculated as the product of the integrated velocity curve and the cross-sectional area of the dorsal aorta. Passive ventricular filling volume equaled dorsal aortic stroke volume multiplied by the fraction of passive filling area, and active ventricular filling volume equaled dorsal aortic stroke volume multiplied by the fraction of the active filling area (Hu et al., 1991).

Fifteen clipped and 15 normal embryos were measured at stage 24. For each embryo, we analyzed five consecutive cycles. The data are presented as means \pm S.E.M., and a statistical analysis was carried out using an unpaired *t*-test. When data were not normally distributed according to the Shapiro–Wilk test, a logarithmic transformation was performed prior to establishing difference between the two study groups. Statistical significance was reached at $P < 0.05$. Calculations were performed with SPSS 10.1 software (SPSS, Inc., Chicago, IL, USA).

Results

The cycle length and heart rate were similar between the two study groups ($P > 0.60$). The mean cycle length was 593 ± 7 ms

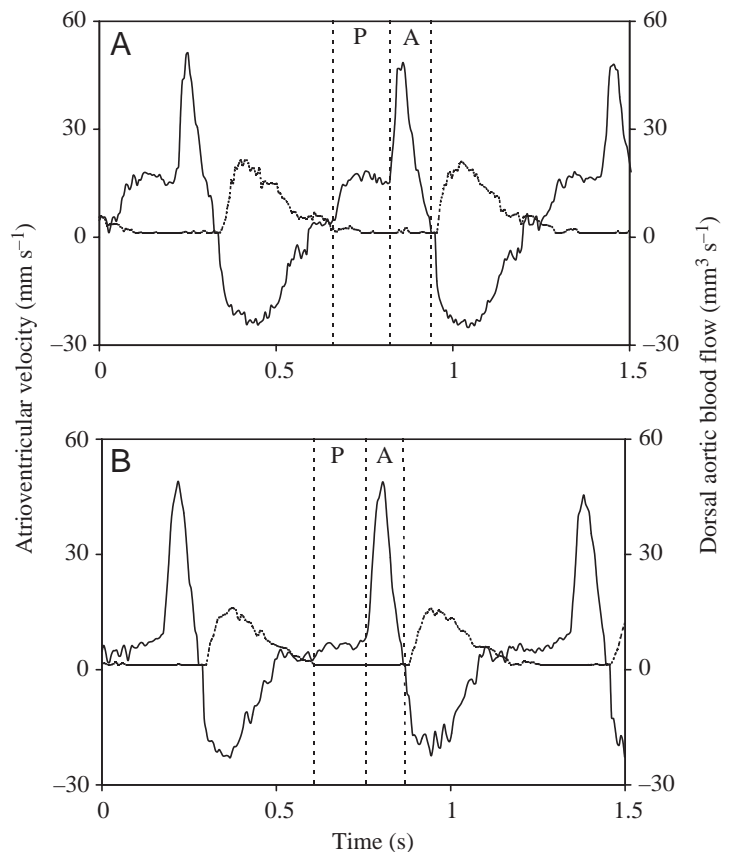


Fig. 1. An example of simultaneously recorded tracings of atrioventricular flow velocity (solid line) and dorsal aortic blood flow (broken line) in a normal (A) and clipped (B) stage-24 chick embryo. Diastole is partitioned into two components: the passive phase indicated by P and the active phase indicated by A.

in the clipped embryo and 597 ± 5 ms in the normal embryo, corresponding to a mean heart rate of 101 ± 1 beats min^{-1} vs 100 ± 1 beats min^{-1} in the clipped and normal embryo, respectively. As a result of this slowed heart rate, the passive and active filling phases of the AV velocity waveform during diastole were well defined (Fig. 1). The AV velocity waveform during ventricular systole coincided with the dorsal aortic flow velocity (Fig. 1).

Dorsal aortic blood flow and stroke volume were similar in the clipped and normal embryos at stage 24 (Figs 2, 3). In the clipped embryos, mean passive ventricular filling volume decreased by 53%, while mean active ventricular filling volume increased 33%, when compared with normal controls (Fig. 3). The passive filling volume accounted for 15% of the stroke volume in the clipped embryos and 33% in the normal embryos. The ratio of passive to active ventricular filling volume was significantly decreased in the clipped embryos compared with normal embryos (0.19 ± 0.02 vs 0.50 ± 0.04) ($P < 0.001$).

Discussion

The present study extends our ongoing investigation of embryonic cardiovascular function after venous clipping experiments at stage 17. We used simultaneous measurements of atrioventricular blood flow velocity and dorsal aortic blood flow to study ventricular diastolic filling characteristics after vitelline vein ligation. Our data of atrioventricular inflow patterns after venous clipping showed a reduced contribution of passive filling in favor of atrial contraction to ventricular filling at stage 24, whereas stroke volume measured in the dorsal aorta was similar between clipped and control embryos at this stage.

Rerouting of venous inflow by permanent obstruction of the right vitelline vein in the early chick embryo results in cardiovascular malformations: ventricular septal defects, valve anomalies and pharyngeal arch artery malformations (Hogers et al., 1997). Results of our hemodynamic study revealed that during the first 5 h after clipping, dorsal aortic blood flow is markedly decreased and is unable to return to baseline values (Stekelenburg-de Vos et al., 2003). However, dorsal aortic blood flow measurements obtained 24 h (stage 21) after clipping demonstrated similar results in clipped and control embryos (S. Stekelenburg-de Vos, unpublished data). We suggest that the reduction in blood flow for at least 5 h may begin a cascade of events that results in the cardiovascular malformations observed later during development.

Proper functional loading is essential for normal cardiac morphogenesis, as the structure and function of the developing heart are intimately linked. Subjecting the chick embryonic heart to mechanically altered loading conditions modifies the myocardial architecture (Sedmera

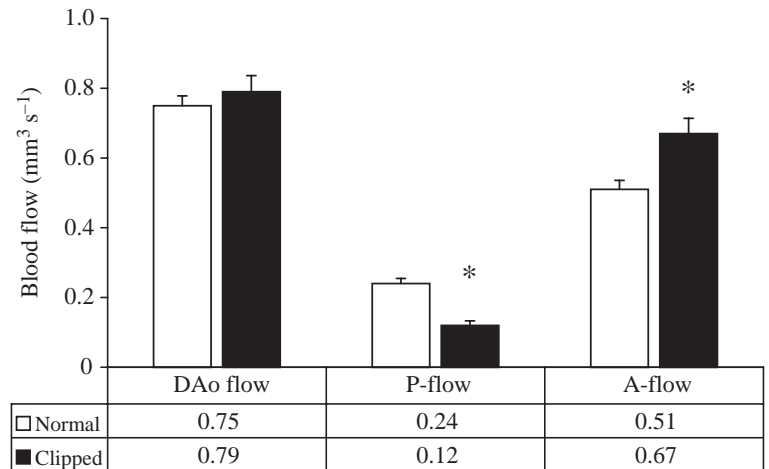


Fig. 2. Dorsal aortic (DAo) blood flow and passive (P) and active (A) components of atrioventricular blood flow measured in normal (open bars, $N=15$) and clipped (filled bars, $N=15$) chick embryos at stage 24. Data are means \pm S.E.M. * $P < 0.003$ vs control.

et al., 1999). Decreased mechanical loading of the left ventricle by left atrial ligation results in reduced levels of proliferation in the left ventricular compact layer and trabeculae (Sedmera et al., 2002). During normal maturation of the heart, myofibrils increase in number and alignment and this will probably affect the myocardial properties of the ventricle (Clark et al., 1986). In the same left atrial ligation model, Tobita et al. (2002) demonstrated an increase in passive stiffness of embryonic myocardium in response to reduced mechanical load. Microtubules, important regulators of cellular organization and fibrillogenesis, seem to be associated with the response of the embryonic myocardium to altered load. An increase in microtubular density and an acceleration of myofiber maturation were observed in the embryonic heart after altered mechanical load and were

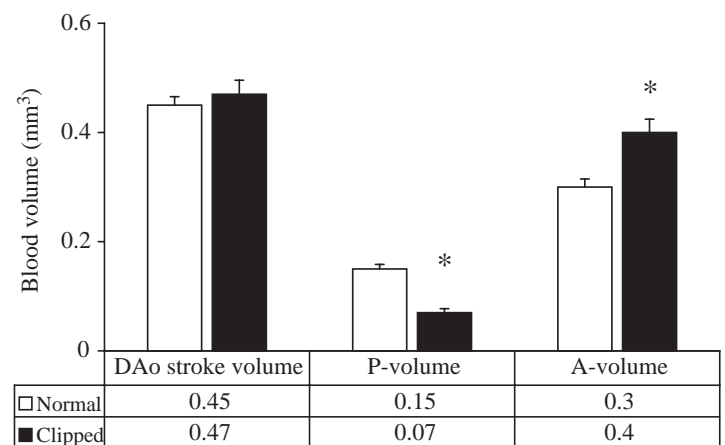


Fig. 3. Dorsal aortic (DAo) stroke volume and passive (P) and active (A) ventricular filling volumes measured in normal (open bars, $N=15$) and clipped (filled bars, $N=15$) chick embryos at stage 24. Data are means \pm S.E.M. * $P < 0.003$ vs control.

related to the increased passive stiffness noted after left atrial ligation (Schroder et al., 2002).

Venous clipping also reduces mechanical load and is likely to modify the myocardial architecture, and this may subsequently result in increased passive stiffness of the embryonic myocardium. This is supported by morphologic examination of venous clipped embryos, demonstrating that, in addition to delayed cardiac looping and impaired cushion formation, the compact layer of ventricular myocardium was thinner and ventricular trabeculation was reduced (Hogers et al., 1998). These morphological changes in developing myocardium could also have an impact on diastolic function and contribute to observed changes in ventricular filling patterns.

By contrast, data of atrioventricular inflow patterns after left atrial ligation showed a reduced contribution of atrial contraction to ventricular filling and a decreased peak velocity from stage 21 to stage 27 (Tobita and Keller, 2000). In the left atrial ligation model, the decrease in volume load is chronic, whereas in the venous clip model the reduction in volume load is temporary. We therefore suggest that the embryonic ventricle responds to temporarily reduced loading conditions by a change in ventricular passive properties, resulting in a reduction in passive filling that is compensated for by an increase of atrial contraction to maintain constant cardiac output.

Experimental disruptions of the venous return by vitelline vein obstruction or left atrial ligation cause cardiovascular malformations (Harh et al., 1973; Hogers et al., 1997). These observations indicate that responses of cardiac tissue to altered biomechanical forces, including blood flow and shear stress, are critical determinants of cardiac development. Also, a role for hemodynamics in modulation of shape and arrangements of endocardial cells in the embryonic chick has been reported (Icardo, 1989). In addition, cultured vascular endothelial cells rearrange their cytoskeletal architecture and change their gene expression profiles in response to flow-induced forces (Davies and Tripathi, 1993; Galbraith et al., 1998; Topper and Gimbrone, 1999; Yoshigi et al., 2003). Thus, experimentally induced flow alterations that translate fluid shear stress to changes in gene expression are likely candidate regulating mechanisms for the response of the developing heart to reduced loading conditions. The hypothesis that fluid shear stress plays an important role in embryonic cardiogenesis was recently substantiated by a study performed in zebrafish embryos. This study describes the quantitative *in vivo* analysis of intracardiac blood flow and shear stress in zebrafish embryos. These data strongly suggest that shear stress forces play some role in the regulation of embryonic cardiogenesis (Hove et al., 2003).

In conclusion, early venous obstruction results in altered diastolic ventricular filling of the stage-24 chick embryo. Our study supports the paradigm that alteration in mechanical loading is a mechanism that can produce changes in cardiac function and structure.

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