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Acutely altered hemodynamics following venous obstruction in the early chick embryo

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Summary

In the venous clip model specific cardiac malformations are induced in the chick embryo by obstructing the right lateral vitelline vein with a microclip. Clipping alters venous return and intracardiac laminar blood flow patterns, with secondary effects on the mechanical load of the embryonic myocardium. We investigated the instantaneous effects of clipping the right lateral vitelline vein on hemodynamics in the stage-17 chick embryo. 32 chick embryos HH 17 were subdivided into venous clipped (*N*=16) and matched control embryos (*N*=16). Dorsal aortic blood flow velocity was measured with a 20 MHz pulsed Doppler meter. A time series of eight successive measurements per embryo was made starting just before clipping and ending 5 h after clipping. Heart rate, peak systolic velocity, time-averaged velocity, peak blood flow, mean blood flow, peak acceleration and stroke volume were determined. All hemodynamic parameters decreased acutely after venous clipping and only three out of seven parameters (heart rate, time-averaged velocity and mean blood flow) showed a recovery to baseline values during the 5 h study period. We conclude that the experimental alteration of venous return has major acute effects on hemodynamics in the chick embryo. These effects may be responsible for the observed cardiac malformations after clipping.

Key words: chick embryo, Doppler ultrasound, hemodynamics, blood flow velocity, venous clip model.

Introduction

The earliest stage at which the beating embryonic human heart can be visualised is the sixth week of gestation, using ultrasonography. Detailed analysis of fetal cardiac and extracardiac flow velocity waveforms using combined high-resolution, two-dimensional ultrasound and Doppler techniques is possible after 8–9 weeks (Splunder van et al., 1996). The most essential events in human cardiovascular development, however, take place between 3 to 8 weeks of gestation, during which the embryonic heart develops from a muscle-wrapped tube into the septated, four-chambered heart, so animal models are required to study mechanisms of early cardiovascular development.

The chick embryo has been used as a model for many decades because in many aspects the embryonic chick heart resembles the developing human heart (Clark and Hu, 1982; Nakazawa et al., 1988). An intervention model for the chick embryo was designed to obtain insight into the long-term hemodynamic effects of altered venous return patterns on cardiac morphogenesis and malformations. Specific cardiac malformations were induced by permanently obstructing the right lateral vitelline vein with a microclip (venous clip model; Hogers et al., 1997), thereby altering the intracardiac blood flow patterns. A spectrum of outflow tract anomalies can be induced by this intervention. Hogers et al. (1997) postulated that alterations in hemodynamic parameters could lead to changes in shear stress, which could alter the expression of shear-stress-responsive genes with downstream alterations in developmental processes, resulting in cardiac malformations. The observation of intracardiac blood flow pattern alterations during clipping, visualised by injected India ink, suggested that hemodynamics is influenced by clipping (Hogers et al., 1999). Other studies have also shown that alterations in hemodynamics can precede the onset of structural defects (Stewart et al., 1986).

A previous study from our group demonstrated that at stage 34 (day 8 of incubation) heart rate was decreased in embryos that had been clipped at stage 17 (stages according to Hamburger and Hamilton, 1951), whereas peak systolic and mean systolic velocities, as well as peak and mean blood flow, were increased compared to normal embryos (Broekhuizen et al., 1999). These results showed the presence of long-term hemodynamic changes after clipping. However, at this point (stage 34) cardiac malformations are already present, so it is impossible to discriminate between the effects of cardiac

malformations and any direct effects of clipping on hemodynamics.

By a modification of the method used for Doppler frequency detection it is now possible to obtain good quality waveforms directly after clipping at stage 17 (Ursem et al., 2001). This allows examination of the direct effects of clipping on hemodynamics, to improve our understanding of how cardiac anomalies may arise following venous obstruction. We hypothesise that cardiac malformations induced by clipping are caused by instantaneous changes in blood flow through the heart resulting in altered activation of shear-stress-responsive genes. In the present work we show the instantaneous effects of clipping the right lateral vitelline vein on hemodynamics in the stage 17 (52–64 h of incubation) chick embryo.

Materials and methods

Animals

Fertilized white Leghorn chick *Gallus domesticus* L. eggs were obtained from Charles River Laboratories (Extertal, Germany) and incubated at 37–38°C, with the blunt end up and at a relative humidity of 70–80%. The embryos were exposed by creating a window in the shell and removing the overlying membranes. Only embryos that were at Hamburger and Hamilton stage 17 (HH 17) (Hamburger and Hamilton, 1951), and that showed no bleeding or deformities, were selected. A total of 32 embryos was included. The material was subdivided into venous clipped embryos (N=16) and control embryos (N=16). In the clipped group the vitelline membrane was removed adjacent to the right vitelline vein and a small incision in the yolk sac membrane was made. Subsequently, the right vitelline vein was obstructed with an aluminium microclip (Fig. 1). Cessation of blood flow proximal to the microclip was confirmed under microscopic surveillance. The microclips were made of a 0.2 mm aluminium sheet. All experiments were performed *in ovo*. During the experiments the egg was placed on a thermoelement of 37°C. In between measurements the window in the shell was resealed with tape and the egg reincubated.

Measurements

Eight dorsal aortic blood flow velocity measurements were made per embryo. The first velocity measurement (baseline) was done directly after exposure of the embryo. The second measurement was performed directly after successful clipping of the right vitelline vein at approximately 4 min from the first measurement. For each clipped embryo a control embryo was measured, matched for the exact time interval between the first measurement before clipping and the second measurement directly after clipping. This was important since cooling of the embryo would take place despite the use of a thermoelement, and cooling influences the outcome of the measurements (Nakazawa et al., 1985; Wispé et al., 1983). The third measurement was performed 30 min after clipping, and the fourth to eighth recordings at 1, 2, 3, 4 and 5 h after clipping, respectively. The control embryos were subjected to the same measurement schedule as venous-clipped embryos.

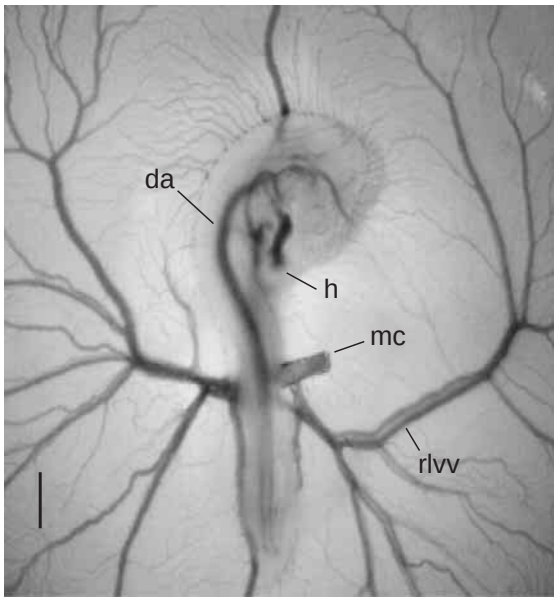

Fig. 1. Stage-17 chick embryo with an aluminium microclip (mc) positioned at the right lateral vitelline vein (rlvv), causing cessation of blood flow proximal to the microclip. h, heart; da, dorsal aorta. Scale bar, 1 mm.

Materials

Dorsal aortic blood flow velocity was measured using a 20 MHz pulsed Doppler meter (model 545C-4, Iowa Doppler Products, Iowa City, IA, USA). The Doppler audio signals were digitised at 12 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). The Doppler probe, consisting of a 750 μm piezoelectric crystal, was positioned at an angle of 45° to the dorsal aorta at the level of the developing wing bud. The sample volume was adjusted to cover the lumen of the dorsal aorta only, excluding other adjacent vessels. The internal aortic diameter was measured at the same level by video imaging using a stereo microscope (model SV 6, Carl Zeiss, Oberkochen, Germany) and a video camera (model SSC-M370CE, Sony Corporation, Tokyo, Japan). The video images were acquired with an image acquisition board (IMAQ PCI-1408, National Instruments, Austin, TX, USA) and subsequently analysed using a custom-built analysis program using IMAQ Vision software (National Instruments). Size calibration in the horizontal and vertical planes of the image was performed by videotaping a scale divided into 10 μm divisions. Aortic diameter was calculated by incorporating the magnified video image displaying the dorsal aorta into the image analysis program.

Blood flow Q was calculated from $Q=\dot{V}\pi d^2/4$, where $\dot{V}$ is mean aortic blood flow velocity and d is the internal aortic diameter. Peak acceleration ($d\dot{V}/dt$) was derived from the dorsal aortic blood flow velocity by means of digital differentiation. Stroke volume was determined from the quotient of the dorsal

aortic blood flow and the heart rate. We determined peak systolic velocity (PSV, mm s^{-1}), time-averaged velocity (TAV, mm s^{-1}), heart rate (beats min^{-1}), peak blood flow (mm^3 s^{-1}), mean blood flow (mm^3 s^{-1}), peak acceleration (mm s^{-2}) and stroke volume (mm^3) for each cardiac cycle. For each embryo a high quality waveform recording of 10 s was used for the analysis. The 10 s recordings contain 20–27 heartbeats. For all hemodynamic parameters in each embryo, the mean of all cardiac cycles was calculated.

Statistical analysis

Reproducibility study

The method by which we obtained the velocity waveform recordings of the dorsal aorta in chick embryos using a 20 MHz pulsed Doppler meter, was validated by conducting a separate study to assess its reproducibility. We measured 10 embryos at HH 17 that showed no bleeding or deformities. All experiments were performed *in ovo*. For each of the 10 embryos, 3 independent measurements were performed at 30 s time intervals. All the measurements were performed by one investigator (S.S.) in the same order: the egg was placed on the thermoelement under the dissecting microscope, the Doppler probe was positioned and the recording was made. In between the measurements the egg was removed from its position. The exact time interval between the measurements was documented. The mean coefficient of variation (CV, %) for heart rate, peak systolic velocity and time-averaged velocity was calculated to determine intraobserver reproducibility. In the calculations, the CV was adjusted for the trend due to cooling over the time points by performing an analysis of variance (ANOVA). A common linear trend was assumed for each embryo in this analysis. The resulting standard deviation (S.D.) of residuals was taken as the S.D. of measurement error. All calculations and statistical analyses were performed using SPSS 10.1 software (SPSS Inc, Chicago, IL, USA).

Hemodynamics

Heart rate, PSV, TAV, peak blood flow, mean blood flow, peak acceleration and stroke volume data are presented as mean ± standard error of the mean (S.E.M.). Hemodynamic parameters were compared within and between groups. Paired *t*-tests were performed within each group to compare mean values at all time points with baseline values. Repeated measurements ANOVA, using SAS PROC MIXED, was performed on the net changes from baseline level to assess whether the profiles of the net changes paralleled each other. The values were standardised by taking the net changes from baseline level in order to adjust for biological variability. Net change profiles in a parallel position indicated a significant difference in the net changes throughout the 5 h study period between the clipped and control groups. If the profiles differed significantly from parallelism, paired *t*-tests were performed at each time point to compare both groups. $P<0.05$ was considered statistically significant.

Statistical analyses were performed using SPSS 10.1 and SAS 6.12 software (SAS Institute Inc, Cary, NC, USA).

Results

Reproducibility study

The mean CV for heart rate, PSV and TAV were 0.9% (range 0.8–1.0%); 3.7% (range 2.8–4.9%) and 4.6% (range 3.0–7.0%), respectively. This indicates a satisfactory intraobserver reproducibility for dorsal aortic waveform derived parameters.

Hemodynamics

The values (means ± S.E.M.) for all the measured parameters in the clipped embryos (N=16) and control embryos (N=16) at all time points are presented in Table 1. The baseline values of all parameters did not significantly differ between both groups. The mean time required to place the microclip was 4 min (range 3.23–4.15 min), and the control group was matched for this 4 min period to differentiate between the influence of cooling of the embryo and clipping. All hemodynamic parameters decreased markedly during the venous clip procedure in the clip group and the 4 min period in the control group.

The profiles of changes from baseline level of all the parameters did not significantly differ from parallelism ($P>0.05$), except for heart rate and peak acceleration ($P<0.05$).

Directly after clipping or the 4 min period (controls), heart rate decreased significantly in both groups (Fig. 2A). This decrease was not significantly different between the groups. However, at 30 min, 1 h and 2 h the difference in heart rate between the clip and control group was significant. In the clipped group it took 3 h for the heart rate to gradually return to baseline level, whereas the control group showed a recovery within 30 min.

The profile of the net changes in the PSV of the clipped group (Fig. 2B) was significantly below the profile of the control group ($P<0.001$). The clipped group demonstrated a 57% decrease in PSV after clipping as compared with a 19% decrease in the control group. This decrease stabilised at 30 min after clipping at ±22%. In the control group PSV was significantly elevated at all time points beyond 2 h compared to baseline level.

The profile of the net changes in TAV of the clipped group (Fig. 2C) was significantly lower than the profile of the control group ($P<0.001$). Following an initially significant decrease of 53% in the clipped group, TAV slowly stabilised at a level of 17% below baseline level. The control group recovered within 30 min and showed a significantly raised TAV at 4 and 5 h.

The profile of the net changes in peak blood flow of the clipped group (Fig. 2D) was significantly below the profile of the control group ($P<0.001$). Peak blood flow decreased by 58% directly after clipping as compared with 18% in the control group. This decrease in the clipped group stabilised significantly below the baseline level at 30 min after clipping. The control group displayed a significantly elevated peak blood flow after 3 h.

The profile of the net changes in mean blood flow of the clipped group (Fig. 2E) was significantly below the profile of the control group ($P<0.001$). In the clipped group mean blood flow was significantly lower directly after clipping, at 30 min

Table 1. *Hemodynamic parameters of stage-17 clipped and control embryos at all measurement points*

		Time						
	Baseline	4 min	30 min	1 h	2 h	3 h	4 h	5 h
Heart rate (beats min^{-1})								
Clipped	147±2	128±2	135±3	138±3	148±4	153±3	151±3	158±3
Control	147±3	131±3	150±2	155±2	157±3	156±3	157±2	159±3
PSV ($mm\,s^{-1}$)								
Clipped	23.0±1.2	9.9±0.6	18.2±1.1	17.9±0.9	17.2±1.2	17.8±1.3	17.6±1.0	17.9±1.2
Control	20.6±1.2	16.6±1.4	20.9±1.5	23.2±1.4	22.7±1.8	23.0±1.5	23.9±1.1	24.3±1.3
TAV ($mm\,s^{-1}$)								
Clipped	6.0±0.5	2.8±0.3	3.6±0.3	3.9±0.3	5.0±0.4	5.0±0.6	4.9±0.4	5.1±0.5
Control	5.1±0.2	3.9±0.3	5.2±0.4	6.2±0.5	6.0±0.6	5.7±0.5	6.2±0.4	6.2±0.4
Peak blood flow ($mm^3\,s^{-1}$)								
Clipped	0.94±0.05	0.40±0.02	0.75±0.06	0.75±0.06	0.72±0.07	0.75±0.08	0.73±0.06	0.74±0.06
Control	0.81±0.05	0.66±0.07	0.82±0.06	0.91±0.06	0.88±0.07	0.90±0.06	0.93±0.04	0.95±0.05
Mean blood flow ($mm^3\,s^{-1}$)								
Clipped	0.24±0.02	0.11±0.01	0.15±0.01	0.16±0.02	0.21±0.02	0.21±0.03	0.20±0.02	0.21±0.02
Control	0.20±0.01	0.16±0.01	0.21±0.02	0.24±0.02	0.23±0.02	0.22±0.02	0.25±0.02	0.24±0.02
Peak acceleration ($mm\,s^{-2}$)								
Clipped	755±42	357±19	516±40	537±26	516±25	517±26	519±26	524±27
Control	720±44	550±40	753±42	781±42	785±44	800±43	803±42	816±43
Stroke volume (mm^3)								
Clipped	0.099±0.007	0.053±0.004	0.064±0.005	0.071±0.007	0.085±0.008	0.084±0.013	0.081±0.007	0.079±0.008
Control	0.082±0.005	0.071±0.006	0.082±0.006	0.094±0.007	0.090±0.010	0.087±0.008	0.094±0.007	0.091±0.006

Values are means ± S.E.M. (N=16 for both groups).
PSV, peak systolic velocity; TAV, time-averaged velocity.

and at 1 h after clipping as compared with baseline level. No significant differences existed at later time points. The control group only showed a significant decrease after the 4 min period and even demonstrated a significantly elevated level of mean blood flow at 4 and 5 h.

The profiles of net changes in peak acceleration (Fig. 2F) significantly (P<0.05) deviated from paralellism. The net changes in peak acceleration of the clipped group and the control group were significantly different at all time points. Within the clipped group peak acceleration was significantly decreased at all time points after clipping as compared with baseline level. The control group showed a significant decrease after the 4 min period only and a significant increase at all time points thereafter.

The profile of the net changes in stroke volume of the clipped group (Fig. 2G) was significantly lower than the profile of the control group (P<0.001). Stroke volume was significantly lower up to 1 h after clipping. At 4 and 5 h stroke volume was also significantly reduced. The control group demonstrated a significant decrease only immediately after the 4 min period.

Discussion

This study describes the instantaneous effects of clipping the right lateral vitelline vein on hemodynamics in the stage-17 chick embryo. The objective was to obtain insight into the direct effects of clipping on hemodynamics using Doppler ultrasonography. Because of the intricate relationship between hemodynamics and morphogenesis, this could improve our understanding as to how cardiac anomalies arise following venous obstruction. In the venous clip model, extraembryonic blood flow is manipulated without direct mechanical interference of the heart such as occurs by altering neural crest migration to the arterial pole of the heart (Kirby et al., 1983) or of the cervical flexure (Manner et al., 1993). Clipping causes a detour of venous inflow to the heart, which alters the intracardiac blood flow patterns, as has been visualised with India ink injections, and induces specific cardiac malformations (Hogers et al., 1997). After clipping, venous blood that is normally drained by the right lateral vitelline vein to the heart is redirected to the posterior vitelline vein *via* a small capillary vessel, which expands into a large anastomosis within a few hours. The alterations in intracardiac blood flow patterns observed immediately after clipping suggest that hemodynamics is influenced by clipping (Hogers et al., 1999). The present study demonstrates that there are major hemodynamic changes during the first 5 h after clipping and we hypothesise about the consequences of these hemodynamic changes in relation to cardiac development.

A significant reduction after clipping is demonstrated for all

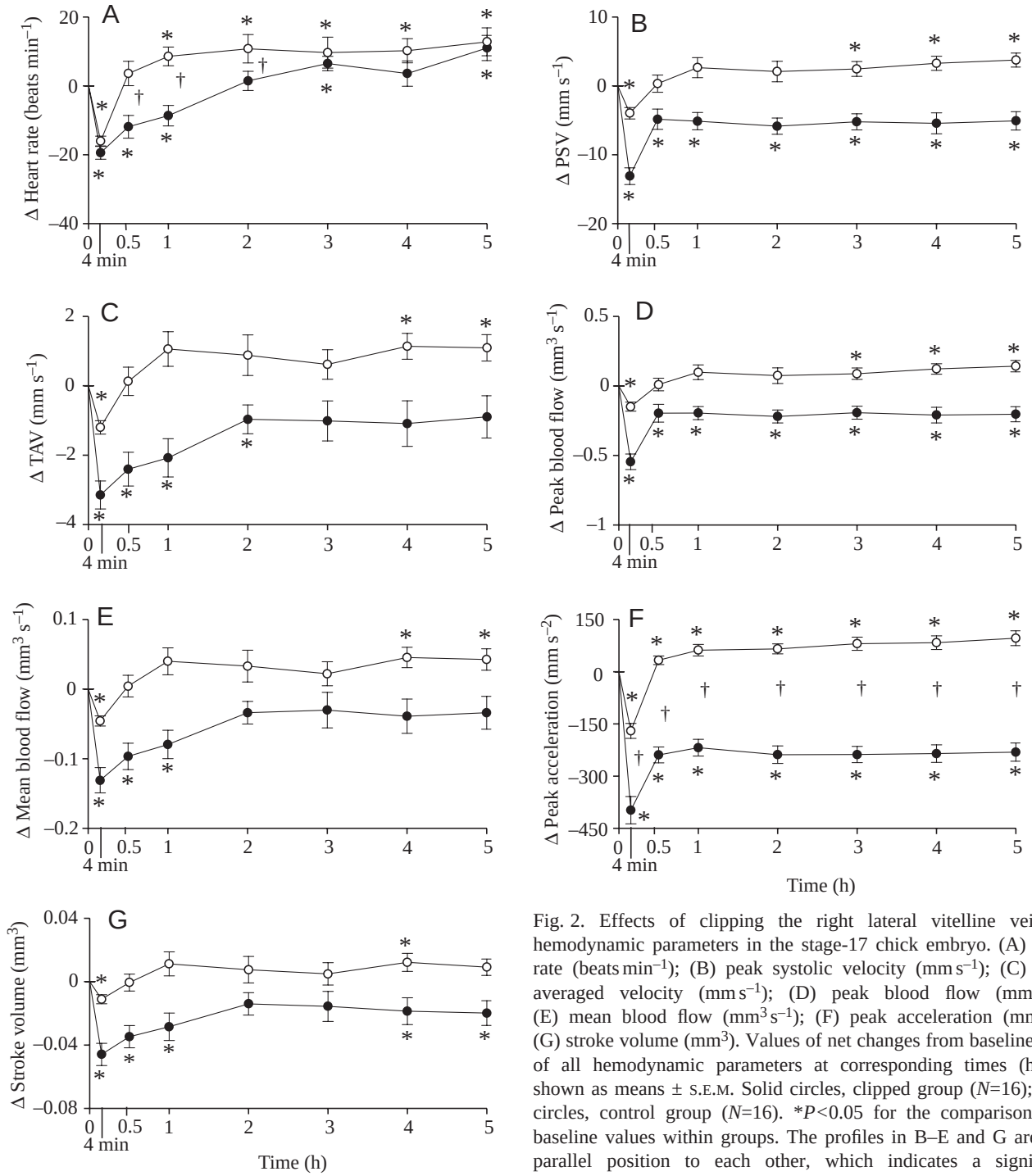

Fig. 2. Effects of clipping the right lateral vitelline vein on hemodynamic parameters in the stage-17 chick embryo. (A) Heart rate (beats min^{-1}); (B) peak systolic velocity (mm s^{-1}); (C) time-averaged velocity (mm s^{-1}); (D) peak blood flow (mm^3 s^{-1}); (E) mean blood flow (mm^3 s^{-1}); (F) peak acceleration (mm s^{-2}); (G) stroke volume (mm^3). Values of net changes from baseline level of all hemodynamic parameters at corresponding times (h) are shown as means ± S.E.M. Solid circles, clipped group (N=16); open circles, control group (N=16). *P<0.05 for the comparison with baseline values within groups. The profiles in B–E and G are in a parallel position to each other, which indicates a significant difference in the net changes from baseline level throughout the 5 h study period. The profiles in A and F do not run parallel to each other, therefore paired t-tests were performed for these parameters to compare values between both groups at the different measurement points, †P<0.05.

hemodynamic parameters, with a recovery seen for only 3 out of 7 parameters (heart rate, time-averaged velocity and mean blood flow) during the 5 h study period. In the control group all parameters recovered to baseline values and displayed an additional increase after 5 h, as could be expected in a normally developing embryo. It is likely that most if not all hemodynamic parameters in the clipped group would recover within the next stages of development, especially since Broekhuizen et al. (1999) observed no significant differences in hemodynamic parameters at stage 24 between clipped and control embryos, except for a lower peak acceleration.

In the clipped group it was evident that the magnitude of decrease of the hemodynamic parameters observed at the first measurement after clipping was mainly due to the clipping,

with additional effects due to cooling. In the control group the observed hemodynamic changes after the 4 min period were due to minor handling and to cooling of the embryo. It is known that chick embryo hemodynamics is greatly influenced by temperature (Nakazawa et al., 1985, 1986; Wispé et al., 1983). Prolonged incubation at low temperatures can even give rise to cardiac malformations (de la Cruz et al., 1966). de la Cruz et al. (1966) observed cardiac malformations in 18.8% of the embryos that had survived during continuous incubation at 35.8°C until hatching. In the clip study by Hogers et al. (1997) the clipped embryos were only subjected to cooling for approximately 4 min, as were our embryos. In their study, specific cardiac malformations were induced in 64% of the clipped embryos, which is much higher than the 18.8% cardiac malformations observed by de la Cruz et al. (1966) during continuous incubation at a low temperature. This supports the conclusion of Hogers et al. (1997) that the cardiac malformations observed by them are caused by obstruction of the right lateral vitelline vein.

When considering the first 30 min of the study period the clipped group was characterised by a significant drop in all hemodynamic parameters whereas in the control group there was a return to baseline level at 30 min. The clipped group also displayed a significantly larger decrease in each hemodynamic parameter as compared with the control group. The changes in the hemodynamic parameters during the first 30 min may be explained as follows. Blood, circulating in the segment of the yolk sac vascular bed that is normally drained by the right lateral vitelline vein, is blocked due to clipping and can therefore not return directly to the heart. Blood is captured in the vitelline vascular bed while the heart keeps on pumping blood into all segments of the vascular bed. Therefore, the actively circulating blood volume is decreased, which results in a drop in venous return or preload and subsequently cardiac stroke volume. The decrease in preload also results in a decrease in peak acceleration, reflecting cardiac contraction force.

During the remainder of the study period, observations in the clipped group showed a full recovery for time-averaged velocity and mean blood flow 5 h after clipping. All other parameters demonstrated a partial recovery that, however, remained significantly below baseline level. From these observations it can be assumed that the embryo compensates to maintain a steady blood supply to all organs. With the development of the vitelline vascular bed, vascular resistance and impedance decreases (Hu and Clark, 1989; Wagman et al., 1990; Yoshigi et al., 1997). We therefore postulate that when a part of the vitelline vascular bed is closed off from the rest of the vascular bed, the vascular resistance will rise. Even when the anastomosis is formed that permanently provides a detour of blood flow towards the heart, the venous vascular resistance will remain elevated because blood has to pass along a longer route to reach the heart. There is a new steady state in the hemodynamic situation that at least meets the demands of the embryo that are necessary to survive 5 h after clipping. When comparing the clipped and control group, all hemodynamic parameters of the latter are situated well above the baseline, except for stroke volume. These findings probably reflect embryonic growth during the study period. As has been described by others, hemodynamic parameters increase with growth of the embryo (Broekhuizen et al., 1993; Hu and Clark, 1989).

The effects of venous clip on heart rate are more complex. There is an initial drop in heart rate in both the clipped and control groups at 4 min which, however, is not significantly different between the groups. This suggests a cooling effect rather than a clipping effect. As outlined above, clipping causes an acute decrease in preload. It is known that heart rate does not respond to acute alterations in loading conditions (Keller et al., 1994). However, at 30 min to 2 h after clipping heart rate remained significantly lower than in control embryos. This suggests a direct influence on the pacemaker function of the heart that prevents a quick and normal heart rate recovery. The cardiac autonomic nervous system is not functional until HH 41, which rules out any impact of this system on heart rate (Higgins and Pappano, 1981; Pappano, 1977). Nevertheless, there is evidence of the presence of β-adrenergic receptors in the myocardium at day 2–2.5 of incubation and circulating neurohumoral agents (Lipshultz et al., 1981). The sudden and persistent decrease in preload following clipping may trigger a release of neurohumoral agents or a change in sensitivity for these agents, resulting in a temporary decrease in heart rate.

The question arises as to how these hemodynamic changes relate to the development of cardiac malformations. During normal cardiac development, cardiac performance increases (Clark and Hu, 1982; Clark et al., 1986) and results in gradual changes in shear stress that either up- or downregulate the expression of shear-stress-responsive genes (Malek et al., 1999; Topper and Gimbrone, 1999). *In vitro* studies have demonstrated that endothelial cells are subjected to fluid shear stress as a result of blood flow and are aligned in the direction of the flow (Malek and Izumo, 1996; Malek et al., 1999) and that changes in shear stress especially cause alterations in gene expression (Fisher et al., 2001). Shear stress depends directly on volume flow (Goldsmith and Turitto, 1986). When major alterations occur in one of the hemodynamic parameters that directly influence the shear stress, altered expression of shear-stress-responsive genes with secondary effects on cardiac development can be expected. Other studies have also shown that alterations in hemodynamics can precede the onset of structural defects (Stewart et al., 1986). The marked decrease in mean blood flow observed in this study may lead to an acute decrease in shear stress, which could be responsible for the induction of cardiac malformations by altering shear-stress-responsive gene expression.

In summary, we conclude that obstruction of the right lateral vitelline vein results in major hemodynamic changes during the first 5 h after clipping. We suggest that these hemodynamic changes induce cardiovascular malformations by altering normal patterns of shear-stress-responsive gene expression.

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