DEVELOPMENTAL DYNAMICS 213:50–58 (1998)

# Diminished Growth of Atrioventricular Cushion Tissue in Stage 24 Retinoic Acid-Treated Chicken Embryos

HANNIE G.A. BOUMAN,[1] MONIQUE L.A. BROEKHUIZEN,[2] A. MIEKE J. BAASTEN,[1]
ADRIANA C. GITTENBERGER-DE GROOT,[1] AND ARNOLD C.G. WENINK[1]*
[1]*Department of Anatomy and Embryology, Leiden University, Leiden, The Netherlands*
[2]*Department of Obstetrics and Gynaecology, Erasmus University of Rotterdam, Rotterdam, The Netherlands*

***ABSTRACT*** **Stage 34 chicken hearts have shown a spectrum of looping disturbances, changed hemodynamics, and changed growth of both right ventricular myocardium and atrioventricular cushion tissue after retinoic acid treatment. To obtain more information about the onset of the malformations we studied stage 24, the stage between the previously studied stage 34 and the moment of treatment. Sixteen stage 24 chicken embryos were examined after treatment with 1 μg all-trans retinoic acid at stage 15 and compared with 6 sham operated embryos. Morphological examination was supported by graphic reconstructions. Absolute volumes of atrial, atrioventricular, and ventricular myocardia were measured by a point counting method. The absolute volumes of the endocardial cushions were measured as well. Fifteen (15/16) retinoic acid-treated hearts did not show marked malformations as far as could be detected with our current macroscopic and microscopic techniques. One (1/16) retinoic acid-treated heart showed an abnormal tubular C-shape with a less bended inner curvature and with an abnormal horizontally oriented atrioventricular canal. The dorsal cushion tissue of this atrioventricular canal was discontinuous with the dorsal mesocardium and covered the malpositioned myocardial border between the atrium and the atrioventricular canal. The volume measurements did show a difference between retinoic acid treatment and sham operations. The retinoic acid-treated hearts showed a significant volume decrease of the atrioventricular cushions. No significant differences were found in the volumes of the ventricular myocardium compared to the sham operated embryos. We hypothesize that, between stages 15 and 24, retinoic acid directly affects the myocardial wall and the cushion tissue formation. In the present material this has resulted in decreased atrioventricular cushion growth, in changed hemodynamics, and in a severe looping disturbance of one embryo. We further hypothesize that, between stages 24 and 34, the malformations with minor looping disturbances will become apparent. Thus, development beyond stage 24 would result in the spectrum of looping disturbances as has been found at stage 34. These latter morphological malformations would lead to increasing hemodynamic changes, resulting in changes in growth as a secondary effect.** ***Dev. Dyn. 1998;213:50–58.*** 

**Key words: heart development; stereology; volume; chick embryo; all-trans retinoic acid; myocardium; cushion tissue**

## INTRODUCTION

To study hemodynamic features of cardiac malformations during early pregnancy and to study normal and abnormal heart development, a chick model has been introduced in which cardiac malformations are induced by retinoic acid (Broekhuizen et al., 1992). We chose retinoic acid, because of its important function in cell differentiation and gene expression on the one hand as reviewed by Ross (1993) and its role in abnormal development when given at high dosage on the other as reviewed by Morriss-Kay (1992). At stage 34 these retinoic acid-treated hearts showed a spectrum of looping disturbances of the heart tube varying from minor to severely malformed (Bouman et al., 1995).

Because in the study by Broekhuizen et al. (1995) hemodynamic changes pointed to myocardial dysfunction, we measured the myocardial volumes of the retinoic acid-treated hearts at stage 34 (Bouman et al., 1997). We found a decreased volume of the right ventricular myocardium only in the severely affected hearts. Another remarkable stereological finding in the severely affected hearts was an increased volume of cushion tissue of the atrioventricular canal. We concluded that this increased growth is a result of a changed morphology and hemodynamics rather than a direct effect of retinoic acid on the cushion tissue. Along with the morphological spectrum, we also found a significant volume decrease of the ventricular myocardium and a significant volume increase of the cushion tissue of the atrioventricular canal from minor to severe malformations.

Grant sponsor: The Netherlands Heart Foundation; Grant number: 900-516-096; Grant sponsor: The Netherlands Organization for Scientific Research.
*Correspondence to: Dr. Arnold C.G. Wenink, Department of Anatomy and Embryology, Leiden University, P.O. Box 9602, 2300RC Leiden, The Netherlands. E-mail: A.C.G. Wenink@Anatomy.MedFac.LeidenUniv.nl
Received 22 October 1997; Accepted 22 May 1998

It is important to know if volume changes are present in the myocardium and cushion tissue of earlier stages. In searching for the earlier malformations caused by retinoic acid treatment we studied the hearts of stage 24 (Hamburger and Hamilton, 1951), the stage between the moment of treatment, i.e., stage 15, and the previously studied stage 34. Besides, at stage 24 there is no parasympathetic innervation of the heart, which rules out parasympathetic disturbances, which may play a role in more advanced stages (Kirby and Stewart, 1983). Retinoic acid-treated stage 24 hearts showed hemodynamic changes, as measured by echo-Doppler at the dorsal aorta level (Broekhuizen, 1996). These changes differed from those at stage 34, but pointed to myocardial dysfunction as well (Broekhuizen et al., 1995). We studied, therefore, the myocardial volumes, including the trabeculae, which play an important functional role in the heart at stage 24 (Challice and Virágh, 1974). The atrioventricular cushion tissue volume in stage 24 was thought to provide more information about the nature of the effect of retinoic acid. A changed volume would make a direct effect on cushion tissue more plausible, as opposed to our previous conclusion that the increased cushion volume is best explained as an indirect effect (Bouman et al., 1997).

Another aspect we are interested in is whether looping disturbances of the heart tube could be found in an earlier stage of development. We divided the looping process into two phases of a continuous process around stage 24. We previously suggested that it is during the second phase of the looping process that the malformations developed (Bouman et al., 1995).

### Terminology of the Embryonic Heart

In normal heart development, different segments can be classified before septation, i.e., atrium, ventricular inlet and outlet segments, and truncus arteriosus (Wenink, 1987; Gittenberger-de Groot et al., 1995). These segments are connected to one another by transitional zones, i.e., the sinu-atrial transition, the atrioventricular canal, the primary fold, and the distal outlet segment. In both the atrioventricular canal and the distal outlet segment, cushion tissue covers the myocardium. At these transitional zones the atrioventricular and semilunar valves develop. The myocardium of both the inlet segment and the proximal part of the outlet segment consists of trabeculae connected to the myocardial free wall. Between the inlet and outlet segments the primary fold develops and becomes a part of the interventricular septum in the completely septated heart. The inlet segment becomes primarily the future left ventricle and the outlet segment becomes primarily the future right ventricle. The distal outlet segment develops into the outflow tract of the left and right ventricles. Because in stage 24 chicken hearts the primary fold is not visible from the outside and is hardly visible on the inside, we did not distinguish between the inlet and the proximal outlet segments.

## RESULTS

### Morphology

In a frontal view (Fig. 1A), gross examination of a normal stage 24 sham heart shows the outflow tract on the right side of the left atrium. The right atrium is positioned posterior to the outflow tract. Right and left atria are connected to the atrioventricular canal, which is recognizable by a groove. This atrioventricular canal is connected to the inlet segment. The primary fold, which is the transition from the inlet to the proximal outlet segment, is not yet present in this stage. The proximal outlet segment is separated from the outflow tract by a groove (Fig. 1A). The outflow tract turns to the left and courses toward the roof of the atria. Cranial to the atria, it turns dorsally and continues as truncus arteriosus, characterized by a mesenchymal wall, toward the pharyngeal arch region.

We examined 16 retinoic acid-treated hearts and compared these with 6 sham operated hearts. One (1/16) heart showed macroscopically a clearly abnormal tubular contour (Fig. 1B). The other 15 hearts (15/16) did not show marked malformations on external and internal inspection. A graphic lumen reconstruction shows the abnormal tubular form more clearly (Fig. 1B). The inner curvature showed less bending than that of the sham operated heart (Fig. 1A, B). The right and left atria were positioned more to the left of the outflow tract and the distance between the right side of the atrioventricular groove and the left side of the groove between the proximal outlet segment and the outflow tract was larger than in the sham. This outer contour was similar to the external appearance of a C-shaped stage 17 chicken heart (Fig. 1D).

A graphic reconstruction of the outer contour of the atrioventricular canal showed that its course in the normal heart was craniocaudal, whereas that of the malformed heart was oriented in a dorsoventral direction (Fig. 2Aa, Ba).

On the inside of the atrioventricular canal of the normal heart two separate cushions were visible which had not yet fused. The ventral cushion tissue covered the ventral wall of the atrioventricular canal (Fig. 2Ab) and was in continuity with the cushion tissue of the outflow tract. The dorsal part covered the atrioventricular myocardium dorsally (Fig. 2Ac) and was in continuity with the mesenchyme of the dorsal mesocardium, forming the caudal border of the sinu-atrial transition and covering cranially the free edge of the atrial septum.

In the abnormal heart the dorsal cushion was discontinuous with the dorsal mesocardium, which resulted in two separate cushions, i.e., the dorsal cushion of the atrioventricular canal and a cushion covering the dorsal wall of the atrium (Fig. 2Bc). The dorsal cushion tissue covered a myocardial fold, which accentuated the border between the atrium and atrioventricular canal (Figs. 2Bd, 3B). In the abnormal heart this myocardial fold was positioned in the frontal plane in contrast to the normal heart, in which the fold is positioned more in the sagittal plane (Fig. 3A, B).

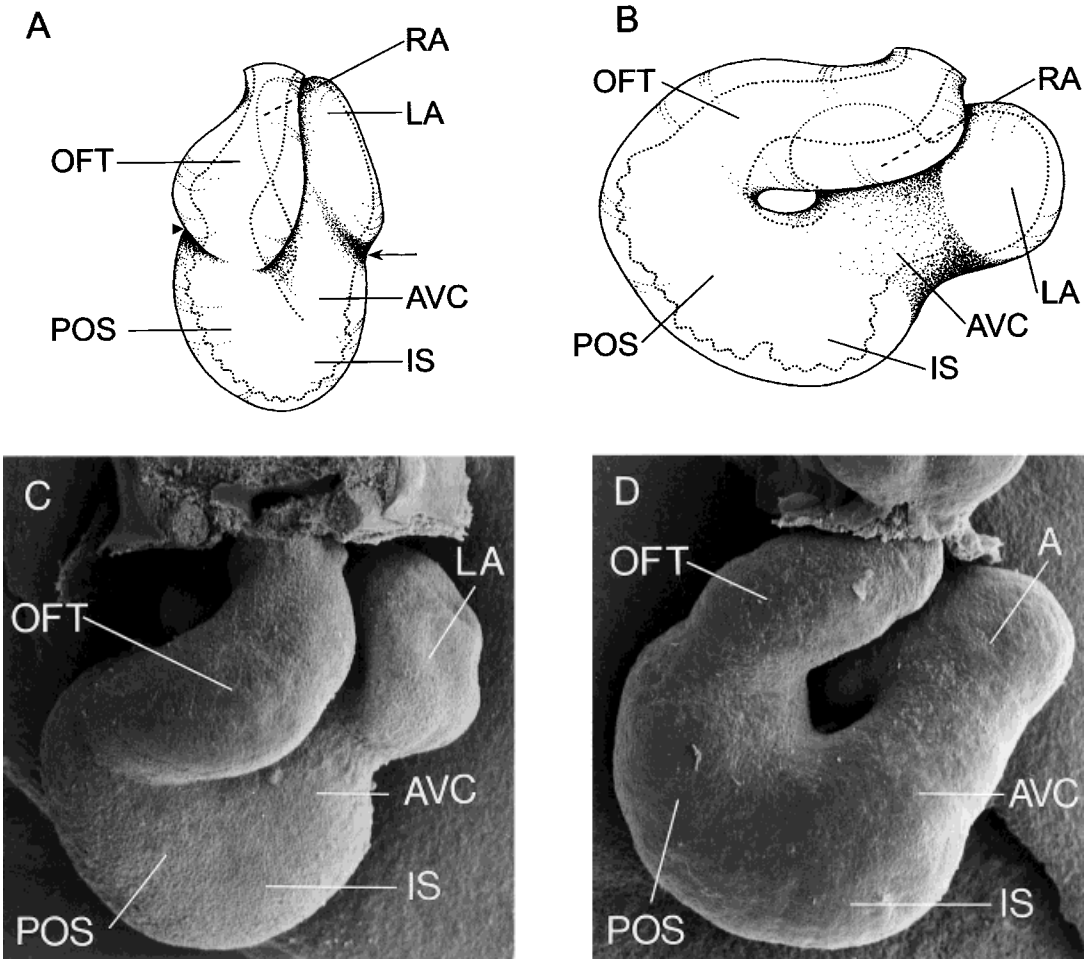


Fig. 1. **A:** Graphic reconstruction of the external contour and the lumen (stippled line) of a sham operated normal stage 24 chicken heart. Frontal view. The right atrium (RA), positioned posterior to the outflow tract (OFT), and the left atrium (LA) are connected to the atrioventricular canal (AVC), which is recognizable by a groove (arrow). The AVC is connected to the inlet segment (IS). The proximal outlet segment (POS) is separated from the OFT by a groove (arrow head). ×45. **B:** Graphic reconstruction of the external contour and the lumen (stippled line) of a retinoic acid-treated stage 24 chicken heart with a tubular form. Frontal view. The inner curvature shows less bending compared to the sham. The left atrium (LA) and right atrium (RA) are positioned more to the left of the outflow tract (OFT) compared to the sham. The outer contour is similar to that of a C-shaped stage 17 heart. ×45. **C:** Scanning electron micrograph of an untreated normal stage 24 chicken heart. Frontal view. ×44. **D:** Scanning electron micrograph of an untreated normal stage 17 chicken heart. Frontal view. Note the C-shape. A, atrium. ×54.

### Stereology

We measured the volumes of the atrial myocardium, the myocardium of the atrioventricular canal, and the total ventricular myocardium (Fig. 4, Table 1). The volumes of the myocardium of the free wall, of the outflow tract, and of the trabeculae were also measured. No significant differences were found between the retinoic acid-treated and the sham operated hearts (Fig. 5, Table 2).

The volumes of the cushion tissue of both the outflow tract and atrioventricular canal were measured as well (Fig. 6, Table 3). The volumes of the atrioventricular cushion tissue were significantly smaller ($P = 0.0487$) than in the sham operated embryos. The single grossly malformed heart had an atrioventricular cushion volume of 0.028 $mm^3$, which is well within 1 S.D. of the retinoic acid-treated mean (Table 3). The volumes of the outflow tract cushion tissue did not differ significantly from those in the shams.

## DISCUSSION

In this study we have examined retinoic acid-treated hearts at stage 24 of development. These hearts show a decreased volume of the cushion tissue of the atrioventricular canal, and unchanged volumes of the myocardium and of the cushion tissue of the outflow tract. One of these hearts shows morphological changes pointing to a looping disturbance.

### Morphology

In previously studied stage 34 hearts we have found that 70.7% (29/41) of those show morphological changes after retinoic acid treatment (Bouman et al., 1995). These hearts have shown a spectrum of looping distur-

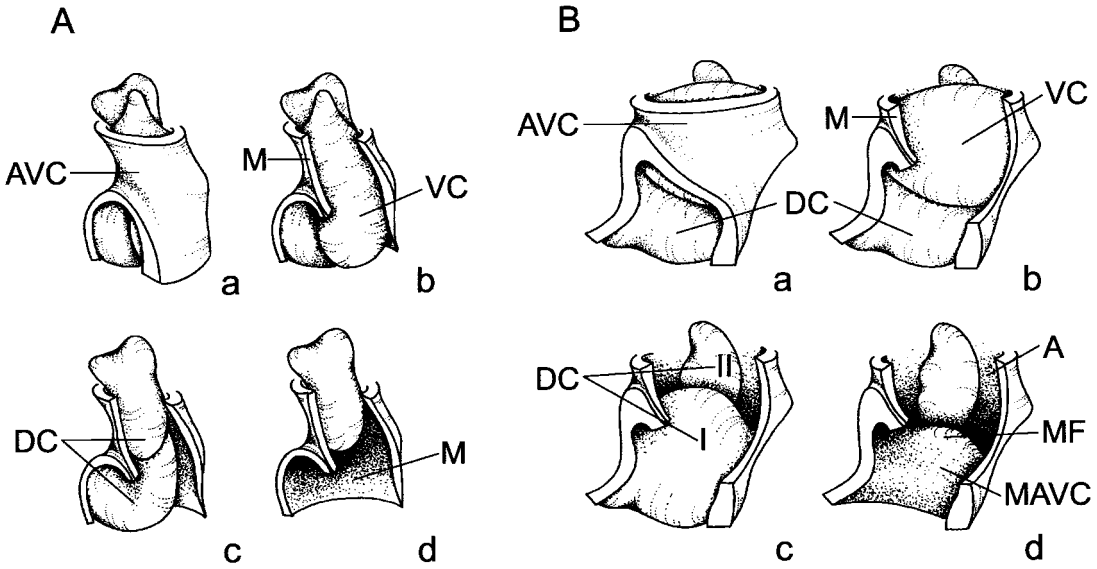


Fig. 2. Graphic reconstructions of the atrioventricular canal (AVC) of stage 24 chicken heart. Frontal view. ×45. **A:** Outer contour of a sham operated embryo with a vertical course of the atrioventricular canal (AVC) (a); ventral cushion tissue (VC) after removal of the myocardium (M) (b); dorsal cushion tissue (DC) after removal of the ventral cushion (c); myocardium (M) of the atrioventricular canal after removal of the atrioventricular part of the dorsal cushion tissue (d). **B:** Retinoic acid-treated heart with C-shape. The outer contour shows a horizontal course of the atrioventricular canal (AVC) (a); ventral cushion tissue (VC) after removal of the myocardium (M) (b); dorsal cushion tissue (DC) after removal of the ventral cushion. The dorsal cushion is discontinuous with the mesocardium resulting in two separate cushions. One is covering the myocardium of the atrioventricular canal (I) (c). The second cushion is covering the dorsal wall of the atrium (II) (c); myocardium of the atrioventricular canal (MAVC) after removal of the atrioventricular part of the dorsal cushion tissue (d). Note the myocardial fold (MF), which accentuates the border between the atrium (A) and atrioventricular canal.

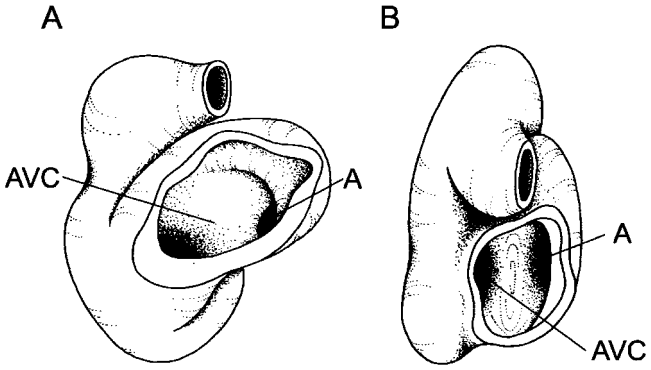


Fig. 3. Left lateral view of the heart atrioventricular canal (AVC) after removal of the left atrium and the dorsal cushion tissue. ×45. **A:** Sham operated embryo. The myocardial fold between the atrium (A) and atrioventricular canal (AVC) is positioned in the sagittal plane. **B:** Retinoic acid-treated heart with C-shape. The myocardial fold is positioned in the frontal plane.

bances in which both inflow and outflow tracts are involved. This spectrum varied from normal hearts with an intact ventricular septum and a normal course of the subaortic outflow tract to severely malformed hearts with a double outlet right ventricle, a horizontal outflow tract, a straddling tricuspid orifice, and a large ventricular septal defect or even a double inlet left ventricle. It should be noted that the absence of malformations as found with the presently used techniques does not confidently point at normality. As was reported previously (Broekhuizen et al., 1995), hemodynamic parameters were statistically significantly different from those obtained from sham operated embryos, even in the absence of a ventricular septal defect.

We have suggested that the looping disturbance predominantly developed during the second stage of the looping process of the heart tube. In the first relevant stage the heart tube bends from a straight tube at stage 11, with venous and arterial poles positioned caudally and cranially in the thorax, via a C-shaped heart tube to a S-shaped loop at stage 24, in which both poles are positioned next to one another (Steding and Seidl, 1980; Männer et al., 1993). The second stage of the looping process starts at stage 24 and results in a wedged position of the arterial pole between the tricuspid and mitral orifices in the completely septated heart at stage 34.

If we compare the results of this study of stage 24 chicken hearts, in which wedging has not yet started, with those of stage 34 hearts, some differences can be noted. We have found only 1 clearly abnormal heart (6.25%) and no spectrum was detectable, i.e., the other 15 hearts (93.75%) did not show marked morphological malformations. These differences suggest that the intermediate malformations as observed in stage 34 become apparent between stages 24 and 34, which is defined by us as the second stage of the looping process. Any pathology that may be present at stage 24 seems to be too subtle to be found with the present investigational approach. We submit that the morphology of the severely affected double outlet right ventricle hearts

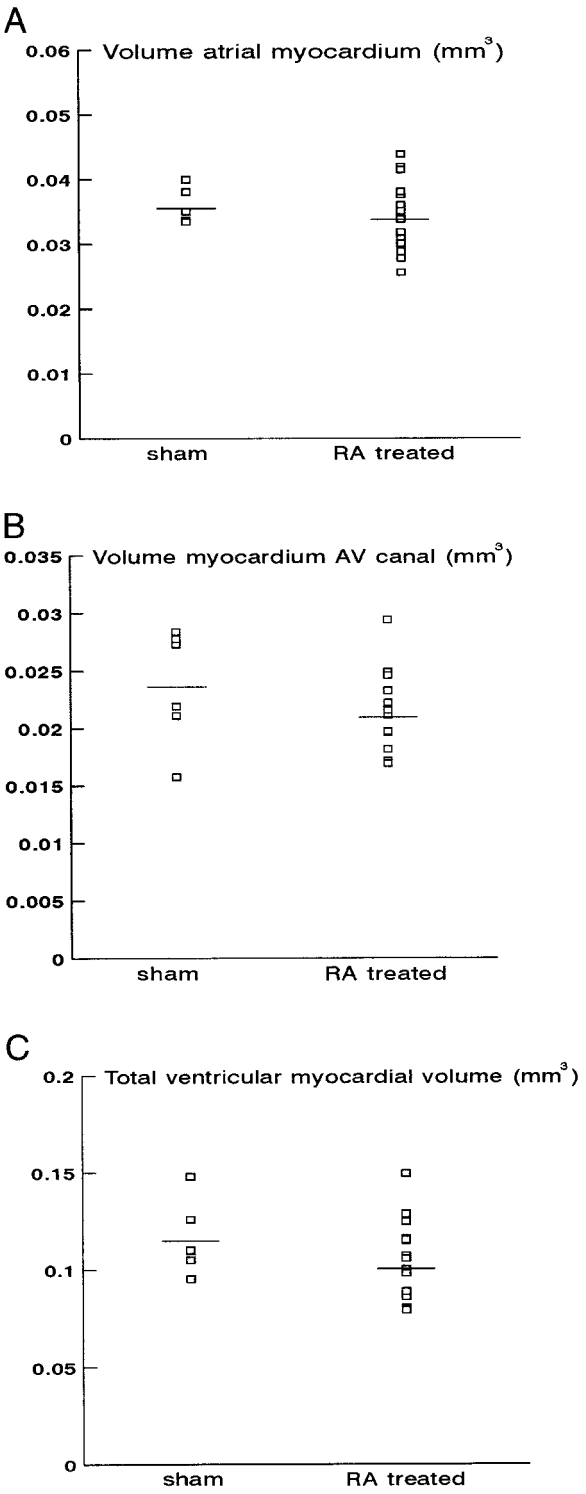


Fig. 4. Myocardial volumes of sham operated hearts and retinoic acid (RA)-treated hearts. No significant differences are shown. **A:** Atrial myocardial volume. **B:** Myocardial volume of the atrioventricular (AV) canal. **C:** Total ventricular myocardial volume.

already becomes apparent between stage 15 (i.e., the stage of treatment with retinoic acid) and stage 24. The abnormal tubular stage 24 heart, in which the inflow tract and outflow tract are positioned apart from one another by a less bended inner curvature, resembles the C-shape of stage 17. This C-shape can also be found in the double outlet right ventricle hearts of stage 34, where the arterial pole is positioned more above the right ventricle, the right and left atria are positioned more above the left ventricle, and the tricuspid orifice is positioned to the left of the arterial pole. Furthermore, this abnormal shape is shown by the absence of the interventricular groove, which is the border between the left and right ventricles. Finally, the apex, which is a part of the left ventricle, of these double outlet right ventricle hearts is more rounded off than is normally seen.

The present material does not rule out the possibility that the finding of 1/16 serious malformation would be incidental. However, the data on later developmental stages have shown (Bouman et al., 1995) that this type of malformation fits in a spectrum of pathology as caused by retinoic acid treatment. For this reason we consider the present finding not to be incidental.

### Stereology

In the study of stage 34 chicken hearts (Bouman et al., 1997) we have found a decreased ventricular myocardial volume in the double outlet right ventricle hearts, caused by a decreased right ventricular myocardial volume and an increased volume of the cushion tissue of the atrioventricular canal. The spectrum of malformations as found in the morphological study was recognized in these volume measurements as a significant trend from the sham to the severely affected hearts. We have concluded that the looping disturbance has caused hemodynamic changes, which have resulted in changes in growth. The looping disturbance was suggested to be caused by a direct effect of retinoic acid on the myocardial wall. This previous study in stage 34 did not support a direct effect of retinoic acid on the cushion tissue.

### Direct Effect of Retinoic Acid on the Myocardial Wall

Several studies have shown that retinoic acid affects the myocardial wall (Taylor, 1979; Hart et al., 1990; Broekhuizen et al., 1995; Pexieder et al., 1995). In the study of Broekhuizen et al. (1995), hemodynamic changes, especially in peak acceleration and stroke volume, are found in stage 34 retinoic acid-treated

**TABLE 1. Mean Volumes and Standard Deviations (S.D.) of the Atrial Myocardium, the Myocardium of the Atrioventricular (AV) Canal, and the Total Ventricular Myocardial Volume of Sham Operated and Retinoic Acid (RA)-Treated Embryos**

| | Mean myocardial volume ± S.D. (mm³) | |
|---|---|---|
| | Sham (n = 6) | RA treated (n = 16) |
| Atrial myocardium | 0.036 ± 0.003 | 0.034 ± 0.005 |
| Myocardium AV canal | 0.024 ± 0.005 | 0.021 ± 0.003 |
| Ventricular myocardium | 0.12 ± 0.02 | 0.10 ± 0.02 |

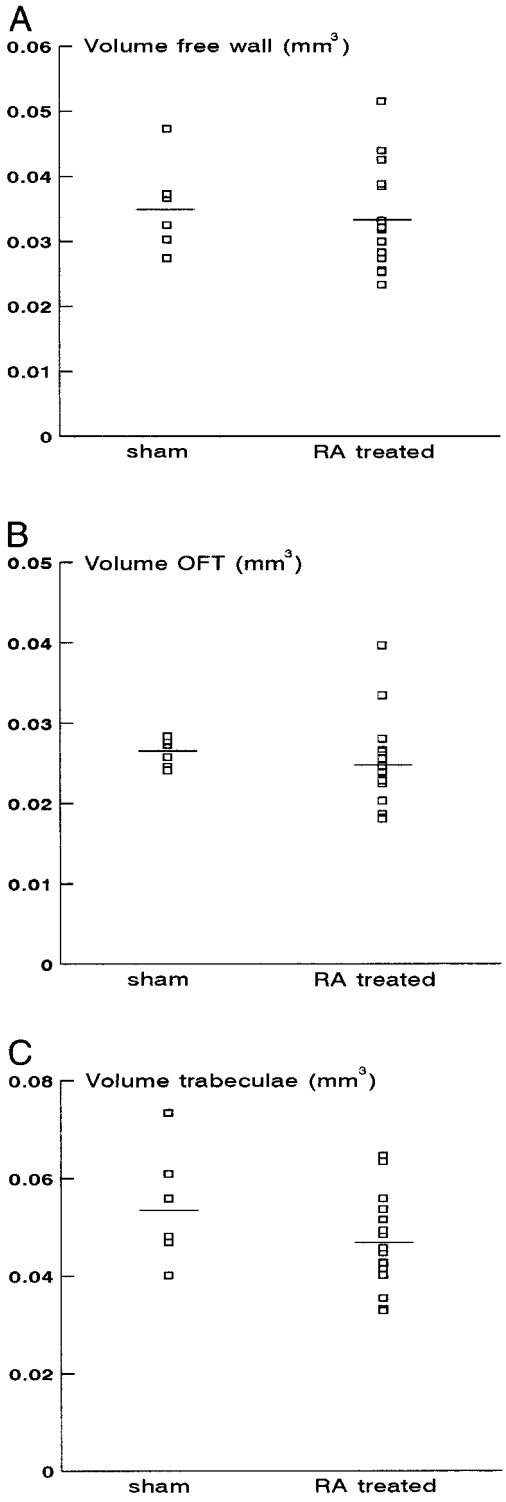


Fig. 5. Myocardial volumes of sham operated hearts and retinoic acid (RA)-treated hearts. No significant changes are shown. **A:** Volume of free wall. **B:** Volume of outflow tract (OFT). **C:** Volume of trabeculae.

hearts. These changes suggest contractile dysfunction. In stage 24 retinoic acid-treated hearts (Broekhuizen, 1996), heart rate was reduced without a compensatory increase of stroke volume. Although this suggests contractile dysfunction as well, it is expressed neither in changes of the total ventricular myocardial volume nor in the myocardial volume of the ventricular segments of stage 24 retinoic acid-treated hearts.

The total ventricular myocardial volume of these stage 24 hearts has not changed compared to the sham operated embryos. This finding is in contrast with a decreased ventricular myocardial volume found at stage 34 hearts, which was caused by a volume decrease of the right ventricular free wall. However, it is consistent with our previously stated hypothesis (Bouman et al., 1997) that the myocardial volume decrease will develop during the second stage of looping, i.e., after stage 24. During the second stage of looping, just before cardiac septation, right ventricular myocardial growth accelerates compared to the left (Knaapen et al., 1995). We have hypothesized that the right ventricular myocardial growth acceleration makes the right ventricle more sensitive to retinoic acid, which subsequently inhibits the myocardial cell proliferation. This hypothesis would only fit if retinoic acid still has its direct effect after stage 24. Another explanation could be that the myocardial cells are affected to the extent that these cells are not able to proliferate. Hierck et al. (1996) attributed this proliferation inhibition to advanced differentiation.

Changed hemodynamics suggest a decreased contraction (Broekhuizen, 1996). Because trabeculations have an important function in contraction at stage 24 (Challice and Virágh, 1974), we expected a changed trabecular volume. In contrast to this expectation, we found an unchanged trabecular volume.

### Direct Effect of Retinoic Acid on Cushion Tissue Formation

Opposite to the increased volume of the atrioventricular cushion tissue of stage 34, stage 24 retinoic acid-treated hearts showed a decreased volume. The only grossly malformed heart did not show an extreme value with respect to the other 15 cases. In the literature several studies on cushion growth have shown a decreased volume of atrioventricular cushion tissue after application of retinoic acid (Davis and Sadler, 1981; Nakajima et al., 1996). These studies strongly suggested a direct effect of retinoic acid on cushion tissue formation. Davis and Sadler (1981) showed a decreased volume of atrioventricular cushion tissue in mouse

**TABLE 2. Mean Volumes and Standard Deviations (S.D.) of the Myocardium of the Ventricular Free Wall of Both Inlet and Proximal Outlet Segments, the Myocardium of the Outflow Tract (OFT), and the Myocardium of the Trabeculae of Both Inlet and Outlet Segments of Sham Operated and Retinoic Acid (RA)-Treated Embryos**

| | Mean myocardial volume ± S.D. ($mm^3$) | |
|---|---|---|
| | Sham (n = 6) | RA treated (n = 16) |
| Myocardium free wall | 0.035 ± 0.007 | 0.033 ± 0.008 |
| Myocardium OFT | 0.026 ± 0.002 | 0.025 ± 0.006 |
| Myocardium trabeculae | 0.05 ± 0.01 | 0.05 ± 0.01 |

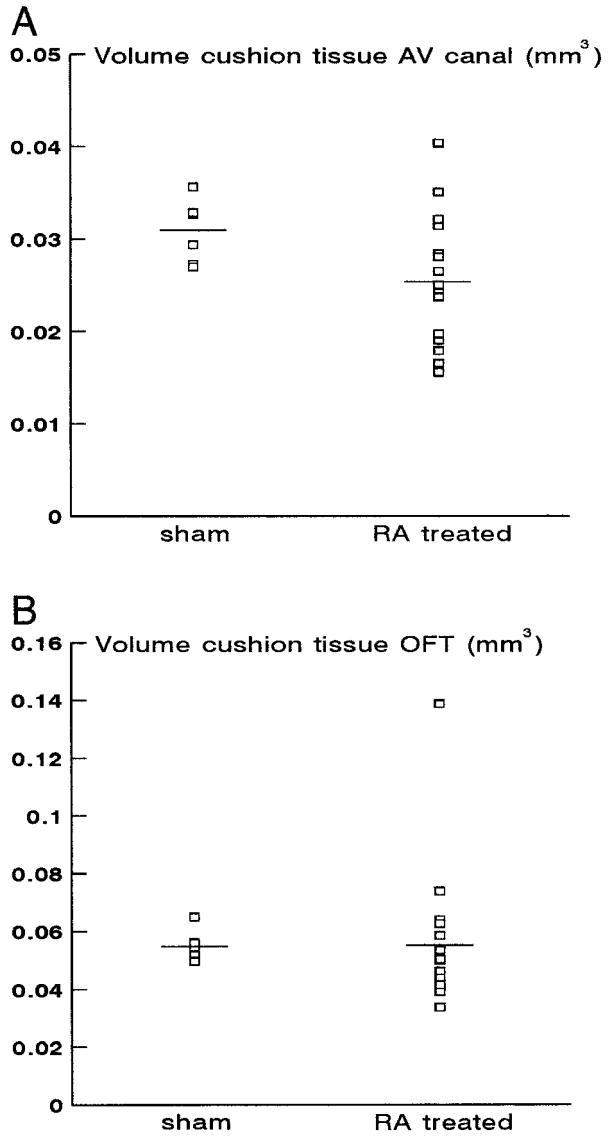


Fig. 6. Volumes of the cushion tissue of the atrioventricular (AV) canal and outflow tract (OFT) of sham operated hearts and retinoic acid (RA)-treated hearts. **A:** Volume of atrioventricular (AV) cushion tissue. The retinoic acid (RA)-treated embryos differ significantly ($P = 0.0487$) from the sham operated embryos. **B:** Volume of outflow tract (OFT) cushion tissue. No significant differences are shown.

embryo explants of day 10.5 (plug day = day 1), which were treated with retinoic acid at day 9.5 and cultured for 24 hr. Nakajima et al. (1996) studied cushion tissue explants of mouse embryos, of which the mothers were treated at day 8.5 (plug day = day 0), and sacrificed at day 9.5. These explants, which were cultured for 48 hr, showed a decreased volume of atrioventricular cushion tissue as well. In these studies and our study, the embryos were treated with retinoic acid during the period of cushion tissue formation (Markwald et al., 1977) in which endocardium will transform into mesenchyme (Markwald et al., 1990). Both treatment and examination were done in the first stage of the looping process of the heart tube. It is conceivable that retinoic acid would act directly on the cushion tissue formation in this first stage of the looping process. However, this disturbed cushion tissue formation could also be caused by an affected myocardium. Myocardium plays an important role as a stimulator in cushion formation (Markwald et al., 1990; Markwald, 1995; Nakajima et al., 1996). Besides, interference of retinoic acid with myocardial structure and function is shown in several studies (Hart et al., 1990; Pexieder et al., 1995; Broekhuizen et al., 1995; Nakajima et al., 1996). Probably, cushion tissue growth is decreased by inhibition of the myocardial stimulus, which is needed for the transition of endothelial cells into mesenchyme (Markwald et al., 1990; Markwald, 1995).

**TABLE 3. Mean Volumes and Standard Deviations (S.D.) of the Cushion Tissue of the Atrioventricular (AV) Canal and the Outflow Tract (OFT) of Sham Operated and Retinoic Acid (RA)-Treated Embryos**

| | Mean myocardial volume ± S.D. ($mm^3$) | |
|---|---|---|
| | Sham (n = 6) | RA treated (n = 16) |
| Cushion tissue AV canal | 0.031 ± 0.004 | 0.026 ± 0.007 |
| Cushion tissue OFT | 0.055 ± 0.005 | 0.06 ± 0.02 |

A decreased volume of atrioventricular cushion tissue could have its effect on hemodynamics. The atrioventricular cushions play an important role as valvular structures in preventing regurgitation (Patten et al., 1948; De la Cruz et al., 1983). It is conceivable that smaller cushions cannot fill up the lumen of the atrioventricular canal, which results in regurgitation. Such regurgitation could then explain the absence of a compensatory increase in stroke volume in retinoic acid-treated hearts showing a reduced heart rate.

## Indirect Pathways

Although hemodynamic changes are shown in stage 24 chicken hearts (Broekhuizen, 1996), these cannot be explained by the type of morphological malformations as were found in stage 34 hearts, because the disturbance in the looping process will predominantly develop afterward, i.e., during the second stage of the looping process.

Thus, the conclusion of stage 34 hearts, in which the looping disturbance is the basis of the hemodynamic changes causing changes in growth, does not hold for stage 24. Here, indirect pathways are less probable, although pathology subtle enough to escape our investigational technique cannot be ruled out.

## Conclusions

We hypothesize that between stages 15 and 24 retinoic acid acts directly on formation of the cushions, probably on the endothelial mesenchymal transformation via the myocardium, which is important in triggering this transformation. This direct effect on the myocardial wall would then also result in hemodynamic changes, and in the most severe cases (one in our study)

in looping disturbances in the first stage, eventually developing into a double outlet right ventricle at stage 34. In the other cases it is to be expected that the morphological disturbance of the endocardial/myocardial balance will lead to second stage looping disturbances, between stages 24 and 34, resulting in the spectrum of minor to severe ventricular septal defects at stage 34. Manifestation of these intermediate morphological malformations and further development of the double outlet right ventricle will result in increased hemodynamic changes, which in turn result in changes in ventricular myocardial and atrioventricular cushion tissue growth at stage 34, as described previously (Bouman et al., 1997).

## EXPERIMENTAL PROCEDURES

Fertilized White Leghorn chicken eggs were incubated until stage 15 (Hamburger and Hamilton, 1951) according to standardized procedures (Broekhuizen et al., 1992; Bouman et al., 1995, 1997). After removal of the shell and the overlying membranes, the chicken embryos were treated with a retinoic acid solution of 1.0 µg all-trans retinoic acid in 1 µl of a 2% dimethylsulfoxide (DMSO) solution, which was applied on the vitelline membrane. In the pilot study of Broekhuizen et al. (1992), it appeared that application of 1 µg retinoic acid at stage 15 results in the highest percentage of abnormal survivors. Afterward incubation was continued until stage 24, i.e., 4 days of development. At this stage, 16 embryos were fixed in Bouin. The hearts were examined macroscopically in order to obtain information about the position of the arterial pole and the atria in relation to the rest of the heart. Then the hearts were embedded in paraffin, serially sectioned at 5 µm thickness, and stained with hematoxylin-eosin for microscopic and stereologic examination. For comparison, 6 sham embryos were treated only with the solvent DMSO under the same conditions as the retinoic acid-treated embryos.

We examined the external position of the truncus arteriosus, distal outlet, and atria in relation to the proximal outlet and inlet segments and their relation to one another. Graphic reconstruction techniques were used to provide three-dimensional insight into the morphology of the heart (Tinkelenberg, 1979). The outer contours were compared with scanning electron micrographs of stage 17 and stage 24 chicken hearts.

In the stereological part of the study we measured the myocardial volumes of the atrium and of the atrioventricular canal. The total ventricular myocardial volume was calculated from the separately measured myocardial volumes of the free wall, the distal part of the outlet segment (also termed the outflow tract), and the trabeculae. We measured the volumes of the cushion tissue of the atrioventricular canal and the outflow tract as well. We used the volume counting method according to Cavalieri (Gundersen and Jensen, 1987). The number of points on a grid hitting 10 sections of the cardiac tissue of interest was counted. The first section was taken randomly and the other sections were taken systematically. From the counted numbers, the volumes could be calculated by Cavalieri's formula:

$$V = \Sigma P \cdot M^{-2} \cdot a \cdot d$$

where V is the volume in $mm^3$, $\Sigma P$ is the total number of counted points on the 10 sections, M is the magnification, a is the point area in $mm^2$, and d is the distance between the counted sections in mm.

The volume estimations of the retinoic acid-treated hearts were compared with those in shams. Statistical analysis was performed by statistical package SPSS (version 5.0). The Mann-Whitney test was used with a significance level of 0.05. The standard deviation of the mean expresses the variability of the volume estimations.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Dr. S. Le Cessie, Department of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands.

## REFERENCES

Bouman HGA, Broekhuizen MLA, Baasten AMJ, Gittenberger-de Groot AC, Wenink ACG. A spectrum of looping disturbances in stage 34 chicken hearts after retinoic acid treatment. Anat. Rec. 1995;243: 101–108.

Bouman HGA, Broekhuizen MLA, Baasten AMJ, Gittenberger-de Groot AC, Wenink ACG. Stereological study of stage 34 chicken hearts with looping disturbances after retinoic acid treatment: Disturbed growth of myocardium and atrioventricular cushion tissue. Anat. Rec. 1997;248:242–250.

Broekhuizen MLA. Hemodynamic evaluation of the preinnervated chick embryonic heart, the retinoic acid model. In: Broekhuizen MLA, ed. Interaction Between Hemodynamics and Abnormal Cardiac Development. Wageningen: Ponsen & Looijen BV, 1996:63–80.

Broekhuizen MLA, Wladimiroff JW, Tibboel D, Poelmann RE, Wenink ACG, Gittenberger-de Groot AC. Induction of cardiac anomalies with all-trans retinoic acid in the chick embryo. Cardiol. Young 1992;2:311–317.

Broekhuizen MLA, Bouman HGA, Mast F, Mulder PGH, Gittenberger-de Groot AC, Wladimiroff JW. Hemodynamic changes in stage 34 chick embryos after treatment with all-trans-retinoic acid. Pediatr. Res. 1995;38:342–348.

Challice CE, Virágh Sz. The phylogenetic and ontogenetic development of the mammalian heart: Some theoretical considerations. Acta Biochim. Biophys. Acad. Sci. Hung. 1974;9(1–2):131–140.

Davis LA, Sadler TW. Effects of vitamin A on endocardial cushion development in the mouse heart. Teratology 1981;24:139–148.

De la Cruz MV, Gimenez-Ribotta M, Saravalli O, Cayre R. The contribution of the inferior endocardial cushion of the atrioventricular canal to cardiac septation and to the development of the atrioventricular valves: Study in the chick embryo. Am. J. Anat. 1983;166:63–72.

Gittenberger-de Groot AC, Bartelings MM, Poelmann RE. Overview: Cardiac morphogenesis. In: Clark EB, Markwald RR, Takao A, eds. Developmental Mechanisms of Heart Disease. Armonk: Futura Publishing Co., 1995:157–168.

Gundersen HJG, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J. Microsc. 1987;147:229–263.

Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J. Morphol. 1951;88:49–92.

Hart RC, McCue PA, Ragland WL, Winn KJ, Unger ER. Avian model for 13-cis-retinoic acid embryopathy: Demonstration of neural crest related defects. Teratology 1990;41:463–472.

Hierck BP, Gittenberger-de Groot AC, van Iperen L, Brouwer A, Poelmann RE. Expression of the β4 integrin subunit in the mouse

heart during embryonic development: Retinoic acid advances β4 expression. Dev. Dyn. 1996;207:89–103.
Kirby ML, Stewart DE. Neural crest origin of cardiac ganglion cells in the chick embryo: Identification and extirpation. Dev. Biol. 1983;97: 433–443.
Knaapen MWM, Vrolijk BCM, Wenink ACG. Growth of the myocardial volumes of the individual cardiac segments in the rat embryo. Anat. Rec. 1995;243:93–100.
Männer J, Seidl W, Steding G. Correlation between the embryonic head flexures and cardiac development. Anat. Embryol. 1993;188: 269–283.
Markwald RR. Overview: Formation and early morphogenesis of the primary heart tube. In: Clark EB, Markwald RR, Takao A, eds. Developmental Mechanisms of Heart Disease. Armonk: Futura Publishing Co., 1995:149–155.
Markwald RR, Fitzharris TP, Manasek FJ. Structural development of endocardial cushions. Am. J. Anat. 1977;148:85–120.
Markwald RR, Mjaatvedt CH, Krug EL, Sinning AR. Inductive interactions in heart development. Role of cardiac adherons in cushion tissue formation. Ann. N.Y. Acad. Sci. 1990;588:13–25.
Morriss-Kay G. Retinoic acid and development. Pathobiology 1992;60: 264–270.
Nakajima Y, Hiruma T, Nakazawa M, Morishima M. Hypoplasia of cushion ridges in the proximal outflow tract elicits formation of a right ventricle-to-aortic route in retinoic acid-induced complete transposition of the great arteries in the mouse: Scanning electron microscopic observations of corrosion cast models. Anat. Rec. 1996; 245:76–82.
Patten BM, Kramer M, Barry A. Valvular action in the embryonic chick heart by localized apposition of endocardial masses. Anat. Rec. 1948;102:299–311.
Pexieder T, Blanc O, Pelouch V, Oštádalová I, Milerová M, Oštádal B. Late fetal development of retinoic acid-induced transposition of great arteries: Morphology, physiology and biochemistry. In: Clark EB, Markwald RR, Takao A, eds. Developmental Mechanisms of Heart Disease. Armonk: Futura Publishing Co., 1995:297–307.
Ross AC. Overview of retinoid metabolism. J. Nutr. 1993;123:346–350.
Steding G, Seidl W. Contribution to the development of the heart. I. Normal development. Thorac. Cardiovasc. Surg. 1980;28:386–409.
Taylor IM. The effect of retinoic acid on the developing hamster heart—An ultrastructural and morphological study. In: Persaud TVN, ed. Advances in the Study of Birth Defects. Vol III. Lancaster: MTP Press, 1979:119–134.
Tinkelenberg J. Graphic reconstruction, micro-anatomy with a pencil. J. Audiovis. Media Med. 1979;2:102–106.
Wenink ACG. Embryology of the heart. In: Anderson RH, Shinebourne EA, Macartney FJ, Tynan M, eds. Paediatric Cardiology. Edinburgh: Churchill Livingstone, 1987:83–107.